Department of Women's and Children's Health
Karolinska Institutet, Stockholm, Sweden

Implications of Localization and Transport Regulation of Postsynaptic Membrane Proteins for Synaptic Function and Psychiatric Disorders

Thomas Liebmann

Karolinska Institutet

Stockholm 2013

Published by Karolinska Institutet. Printed by Larserics

ISBN 978-91-7549-387-9

ABSTRACT

A fundamental means of regulating protein function in cells is through modulation of protein abundance at the sites of action via controlled transport processes. The complexity of neurons makes them especially reliant on regulated delivery of proteins to specialized structures throughout the distant cell branches. Proteins destined for pre and postsynaptic membrane are synthesized within the cell then undergo regulated transport to the membrane and ultimately to the respective synaptic signaling domains. To date, details of membrane delivery for most types of postsynaptic proteins have been lacking. As moderators of many neuropsychological functions, signaling effects of G protein-coupled receptors (GPCRs) are of major interest to the scientific community. Yet few from this important class of synaptic membrane proteins have been thoroughly examined for trafficking regulation mechanisms.

In the included GPCR study, we have evaluated the postsynaptic transport routes for a number of GPCRs involved in affective and schizoaffective disorders to describe an alternative approach for therapeutic intervention. Bulk mobility measurements with fluorescence recovery after photobleaching (FRAP) in cultured hippocampal neurons describe the common GPCR trafficking pathway via energy-efficient lateral diffusion. One GPCR, the serotonin 1b receptor (5-TH_{1b}), deviated by transporting to dendritic compartments in secretory vesicles. Membrane delivery via exocytosis was detected at preferential sites throughout the dendrite branches before delivery to the synapses via diffusion. These results suggest that unique postsynaptic transport routes may provide novel approaches for selective therapeutic regulation of receptor abundance at the postsynaptic membrane.

The last decade of membrane trafficking research has deepened our understanding of the importance of diffusion regulation for synaptic restructuring and modulation of neurotransmission efficacy. An overlooked membrane protein that is essential for proper neuronal function is the sodium-potassium pump (Na,-ATPase). With the super-resolution methods of structured illumination microscopy (SIM) and photoactivated localization microscopy (PALM), we demonstrated that both neuron-specific alpha 3 (ATP1a3) and the ubiquitous alpha 1 (ATP1a1) isoforms can form nanoclusters throughout the dendrites and are enriched in excitatory spines. Using PALM for molecular quantification, we estimate many hundreds of pumps per spine.

To understand how dynamic this picture of pump abundance is in the living neuron, we performed a detailed single particle trafficking (SPT) study on postsynaptic ATP1a3. With quantum dot labeling of single molecules, we revealed high extrasynaptic diffusion and reduced mobility at excitatory synapses. Inducing or inhibiting synaptic activity by chemical methods revealed that the pump diffusion is highly regulated and differentially responsive to varying synaptic activation.

With the novel view of regulated pump diffusion at and around synaptic membrane, we assessed the potential for physiological regulation of pump mobility via extracellular interactions. We demonstrated that most membrane-bound ATP1a3 are maintained in an oligomer with the non-enzymatic beta subunit of the pump which contains a large extracellular domain. Furthermore, addition of a soluble peptide, known to bind with the beta subunit, significantly reduced the synaptic mobility of the pump. These studies describe regulation of postsynaptic membrane protein trafficking that adds to our fundamental knowledge of neuronal function and introduces new avenues for signal regulation.

LIST OF PUBLICATIONS

I. **Thomas Liebmann**, Markus Kruusmägi, Nermin Sourial-Bassilious, Alexander Bondar, Marc Flajolet, Paul Greengard, Lena Scott, Hjalmar Brismar, Anita Aperia.

A noncanonical postsynaptic transport route for a GPCR belonging to the serotonin receptor family.
Journal of Neuroscience. 2012, Dec 12; 32 (50): 17998-8008.

II. **Thomas Liebmann**, Markus Kruusmägi, Alexander Bondar, Hjalmar Brismar, Anita Aperia.

Diffusion dynamics of neuronal Na,K-ATPase revealed by single particle tracking.
Manuscript.

ARTICLE APPENDIX

AI. **Thomas Liebmann**, Hans Blom, Anita Aperia, Hjalmar Brismar.

Nanoscopic elucidation of Na,K-ATPase isoforms in dendritic spines.
Optical Nanoscopy. , 2013, Nov 9; 2:6.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine, serotonin
AHC	Alternating hemiplegia of childhood
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANG-2	Asante NaTRIUM Green-2
ATP	Adenosine triphosphate
ATP1a1	Na,K-ATPase alpha 1 subunit
ATP1a3	Na,K-ATPase alpha 3 subunit
ATP1b1	Na,K-ATPase beta 1 subunit
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
DHPG	Dihydroxyphenylglycine
DNA	Deoxyribonucleic acid
EMCCD	Electron-multiplying charge-coupled device
ER	Endoplasmic reticulum
FP	Fluorescent protein
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
IgG	Immunoglobulin G
IP_3	Inositol triphosphate
mGluR	Metabotropic glutamate receptor
MONaKA	Modulator of Na,K-ATPase
MSD	Mean square displacement
Na,K-ATPase	Sodium-potassium adenosine triphosphatase
NMDA	N-Methyl-D-aspartic acid
PAFP	Photoactivatable fluorescent protein
PAGFP	Photoactivatable green fluorescent protein
PALM	Photoactivated localization microscopy
PSD-95	Postsynaptic density protein 95
QD	Quantum dot
RDP	Rapid-onset dystonia parkinsonism
SEP	Superecliptic pHluorin
SIM	Structured illumination microscopy
SPT	Single particle tracking
SSRI	Selective serotonin reuptake inhibitor
STED	Stimulated emission depletion
STORM	Stochastic optical reconstruction microscopy
TTx	Tetrodotoxin
WT	Wild type

1 BACKGROUND

1.1 STRUCTURE AND FUNCTION OF NEURONS

The brain is an immensely complex organ, by far the most complex in the human body. Despite the more than 100 years since Ramón y Cajal was awarded the Nobel Prize in Physiology or Medicine (1906) for his pioneering description of the nervous system, we are still only scratching the surface of how the brain works to provide the rather abstract concepts like thought and memory that each individual experiences. Most of the active signaling in the brain is attributed to the polarized, electrically excitable cells called neurons that generate and receive input from neighboring and distant neurons throughout the brain (Hodgkin and Huxley, 1939). The regulation of these triggered signals between neurons is the basis of network activity and the primary task of the brain (Sherrington, 1947).

Neurons can vary widely throughout the brain, but their basic structure and function remain the same. Each neuron consists of a main body, called the cell body or soma, and extending branches (**Figure 1.1**). The axonal branching is the signal delivery component of the neuron. When a neuron is excitation beyond a threshold level, an action potential is generated near the cell body (Johnston and Roots, 1966, Palay et al., 1968) and rapidly travels down the length of the axon branches where it triggers release of chemical signals (neurotransmitters) to recipient neurons. The second set of branches, called dendrites, is responsible for detecting the released chemicals and transducing the signal to the cell body for possible generation of a new action potential. This highly regulated cascade is the primary purpose of each neuron in the brain.

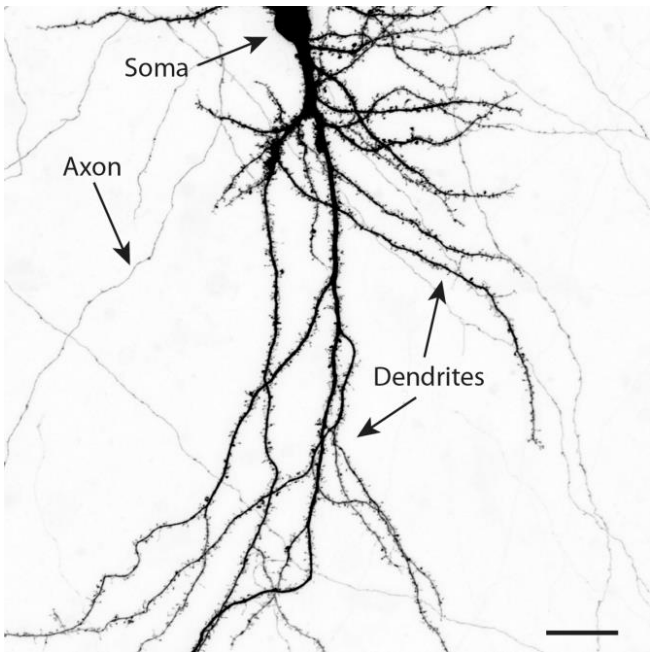

Figure 1.1 Stack projection from confocal images of a fluorescently labeled neuron. The cell body or soma and branches of axons and dendrites are clearly visible. Fluorescence is from activation of transfected yellow fluorescent protein. A description of transfection and fluorescent protein follows below. Scale bar = 25 µm.

The molecule responsible for detection of neurotransmitters released from the axons is appropriately called a receptor. Receptors exist in many forms and have specialized function in response to their activating ligand. Neurotransmitter receptors can be produced in different parts of the cell, but a majority seems to be produced in the cell body, where much of the protein production machinery exists. Production triggers are a key regulatory mechanism of all cellular proteins, but also essential for membrane protein regulation are the trafficking processes that deliver proteins to their site of function. This will be one of the main themes of this thesis. Without proper localization, a protein will not provide its function or it may be active at a position of the cell that is deconstructive to the overall cell function.

The location of focus when discussing neuronal function is most often the point of contact between neurons where the transmitter signal is released and detected, called the neuronal synapse. The synapse consists of the axon terminal, which stores the releasable neurotransmitters, and the dendrite compartment receiving the signal, called the dendritic spine for excitatory synapses (Harris et al., 1992) (**Figure 1.2**). The cleft between the presynaptic (axon) membrane and postsynaptic (dendrite) membrane can be as narrow as 15 nm, thereby maintaining a high concentration of neurotransmitters for receptor activation (Peters et al., 1991).

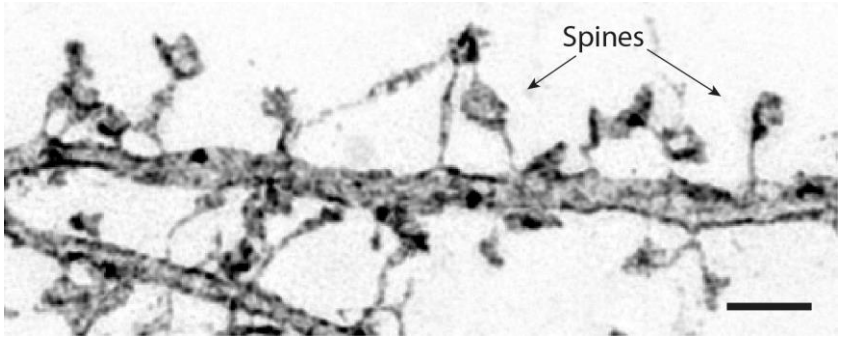

Figure 1.2 Super-resolution image of a membrane protein (ATP1a3) in dendrites. Round objects forming along the main dendrite branches are called spines. Scale bar = 2 µm.

Inhibitory synapses are formed directly on the dendritic membrane and therefore lack spine structures. The fundamental release and response mechanisms, however, still pertain. Because of the highly localized release of transmitters, the arrangement of receptors and the many regulatory membrane and intracellular proteins is an effective means of adapting signal responses. A detailed knowledge of protein trafficking to and from the synapse is essential for the understanding of how neurons modulate their function which impacts the higher order processes like network signaling and even complex brain functions like learning and memory.

1.2 G PROTEIN-COUPLED RECEPTORS

In concert with the diversity of neurotransmitters in the brain, there exists a plethora of receptor types. Each receptor type has further multiplicity of subgroups, splicing variants, etc. The studies in this thesis focus on a small set of receptors included in a group designated as G protein-coupled receptors (GPCRs). Named for their common

capacity to bind to G protein signal transducers, GPCRs are membrane proteins with seven trans-membrane helices (Rosenbaum et al., 2009). Aside from these commonalities, GPCRs have a tremendous diversity and respond to many different environmental cues (Kroeze et al., 2003). GPCRs are the essential pathway for cells in our body to sense extracellular triggers such as hormones and neurotransmitters. Much of the early work that led to the 2012 Nobel Prize in Chemistry was performed on adrenergic receptors, but many of the revelations are applicable to other members of the GPCR superfamily. In recent decades, they have been considered the most important therapeutic target in the brain, evident in the clear majority of neurological drugs that are aimed at modulation of GPCRs.

A comprehensive discussion of GPCRs and their respective transmitters is beyond scope of this thesis, but an introduction to a few included receptors will be helpful. The first study in this thesis provides a description of trafficking mechanisms for a small subset of GPCRs, including examples of serotonin, dopamine and glutamate receptors. Each of these neurotransmitters has specialized functions in the brain.

1.2.1 Dopamine

Dopamine is a catecholamine transmitter that is enzymatically generated in the neuron from tyrosine. A major dopamine center in the brain is the substantia nigra, from where it is projected to the striatum to provide motor control (Carlsson, 1959, Zeiss, 2005). Production in the ventral tegmental area is delivered to multiple areas, including nucleus accumbens and the cortex, where it governs the reward system of the brain (Bjorklund and Dunnett, 2007). Improper regulation of dopamine can contribute to development of numerous diseases (Girault and Greengard, 2004). Dopamine is also involved in learning and memory. Loss of dopamine secretion (death of dopamine producing cells) from the substantia nigra is a principal cause of Parkinson's disease (Girault et al., 1990, Carlsson, 2002). Elevated levels of dopamine transmission in the reward system are associated with drug addiction (Kuhar et al., 1991). Dopamine is also connected with attention deficit hyperactivity disorder and is believed to be involved in psychosis and schizophrenia (Hietala and Syvalahti, 1996, Wu et al., 2012).

Dopamine receptors are divided into two families, the D_1-like and D_2-like receptor families. The D_1-like family includes the D_1 and D_5 receptors, whereas the D_2-like family includes D_2, D_3 and D_4. D_1-like receptors are grouped due to their common G_s-coupling, which describes their mediation of excitatory neurotransmission through stimulation of adenylyl cyclase. D_2-like receptors are G_i-coupled, designating an opposing regulation of adenylyl cyclase and thereby inhibition of cyclic adenosine monophosphate (cAMP) production.

1.2.2 Serotonin

Serotonin (5-HT) is a different type of neurotransmitter, known as a monoamine. It is predominantly found in the gut, but it also plays an essential role in the central nervous system (CNS). The 5-HT network begins where it is manufactured from tryptophan in the raphe nuclei of the brain stem (Hannon and Hoyer, 2008). From there it extends to a majority of the brain to govern an immense number of neurological functions including mood, cognition, sleep and anxiety. The complexity of the serotonin system

has proven a hindrance to the elucidation of disruption mechanisms (Berton and Nestler, 2006).

Serotonin has been a major interest of the pharmaceutical industry, owing to its involvement in common depressive disorders (Svenningsson et al., 2006, Lopez-Munoz and Alamo, 2009). There is still dissent regarding the cause of depression, but it is evident that the state of many patients is appreciably improved by the administration of therapeutics. The therapeutic approach can be generalized as an aim to increase exposure to serotonin. Serotonin release from serotonergic neurons is rapidly transported back into the cell as is true for all other neurotransmitters. Antidepressants work to prevent reuptake of serotonin, hence the name selective serotonin reuptake inhibitors (SSRIs), or to prevent serotonin breakdown by monoamine oxidase inhibitors. Both approaches result in elevated levels of serotonin in the synaptic cleft. Severe restrictions are applied in many countries to some antidepressants, and there is often an age minimum due to the high level of side effects which can include an increased risk for suicidal ideation (Soutullo and Figueroa-Quintana, 2013).

Serotonin receptors are more diverse than the dopamine receptors in that they exist in seven different groups. They are organized by similarity of function and structure. Among the fourteen unique receptors, only 5-HT_3 is an ion channel; the rest of which are GPCRs. Of relevance to this thesis are 5-HT_{1a}, 5-HT_{1b} and 5-HT_4. The 5-HT_1 family is G_i-coupled, whereas 5-HT_4 is G_s-coupled, again demonstrating the diverging receptor-specific transmission response even within each receptor type.

1.2.3 Glutamate

Unlike dopamine and serotonin, glutamate is an example of an amino acid neurotransmitter. Considered a non-essential amino acid because of its synthesis in neurons, glutamate is the prominent excitatory neurotransmitter in the CNS (Meldrum, 2000). Glutamate release from presynaptic cells induces activity in the responding postsynaptic cells; a process which occurs throughout the brain. Excessive exposure or sensitivity to glutamate can contribute to uncontrolled, elevated cellular and network activity and ultimately neuron toxicity or induced seizures (Camacho and Massieu, 2006). Improper regulation can lead to numerous diseases including Alzheimer's and Huntington's disease as well as disrupted mental development (Calabresi et al., 1999, Pogocki, 2003, Bear et al., 2004).

Glutamate receptors can be grouped into two basic types: ionotropic (ligand-gated ion channels) or metabotropic (ligand-gated signal transducers). Ionotropic glutamate receptors (iGluRs) are tetrameric membrane proteins and compositions are highly regulated for modulation of ion selectivity (Rosenmund et al., 1998, Dingledine et al., 1999, Traynelis et al., 2010). In this thesis there is some discussion of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors, both of which are often discussed in the context of long term changes in synaptic signaling which is the basis for processes of learning and memory (Bear, 1996, Malenka and Bear, 2004). Metabotropic glutamate receptors (mGluRs) function as regulators of various ion channels through activation of their associated G proteins, qualifying them as GPCRs. They are categorized in groups based on their G

protein coupling and agonist affinity similarities: group 1 (mGluR1 and mGluR5), group 2 (mGluR2 and mGluR3), and group 3 (mGluR4, mGluR6, mGluR7 and mGluR8). Group 1 mGluRs are discussed in this thesis. Unlike other included GPCRs, they are G_q-coupled, indicating that activation initiates production of inositol 1,4,5-trisphosphate (IP_3) which subsequently stimulates release of stored intracellular calcium from the endoplasmic reticulum (ER). Group 1 mGluRs are involved in many other complex pathways that can both increase and decrease cell activity. They have been implicated in numerous disorders that include epilepsy, autism and Parkinson's disease (Chapman, 2000, Bear et al., 2004, Marino and Conn, 2006).

A summary of GPCRs included in this study are listed in **Table 1.1**.

GPCR	Transmitter	Receptor	G protein	Response
D_1	dopamine	D_1-like	G_s	Incresed cAMP
D_5	dopamine	D_1-like	G_s	Incresed cAMP
D_2	dopamine	D_2-like	G_i	Decreased cAMP
5-HT_{1a}	serotonin	5-HT_1	G_i	Decreased cAMP
5-HT_{1b}	serotonin	5-HT_1	G_i	Decreased cAMP
5-HT_4	serotonin	5-HT_4	G_s	Incresed cAMP
$mGluR_5$	glutamate	group 1	G_q	Increased IP_3

Table 1.1 GPCRs included in constituent articles of this thesis.

1.3 SODIUM REGULATION IN NEURONS

I won't go into the detail required to fully grasp the complexity of sodium regulation and its function in normal neuronal activity and disease states, but a brief introduction is necessary as a prelude to the studies of neuronal sodium-potassium pumps included in this thesis. A baseline sodium concentration of approximately 10 mmol/L is maintained in the cytosol of neurons (Rose, 2002). This is in contrast to the approximate 150 mmol/L of sodium found in the interstitial fluid outside of cells (Bito and Davson, 1966). This difference in sodium levels is fundamentally essential for the functional electrical properties that contribute to the uniqueness of neurons from other cell types. In general terms, the chemical gradient provides a voltage potential difference between the inside and outside of cells (Kandel et al., 2000). This potential energy is utilized by cells to facilitate controlled transport of ions across the resistant plasma membrane (Wright and Turk, 2004).

Important for all eukaryotic cell types, the maintenance of low sodium establishes the resting potential across the plasma membrane. This is a product of the equilibrated chemical and electrical (charge) distribution. In neurons, the resting membrane potential is typically below -70 millivolts (Kandel et al., 2000). Changes of ion concentration across the membrane locally alter the membrane potential, which can trigger voltage-sensitive membrane proteins (voltage-gated ion channels) to open and allow passive exchange of ions across the membrane. This ion flux can increase or decrease the membrane potential according to the ion charge and transfer direction. Consequently additional ion channels can be opened and closed according to the altered voltage difference or detected concentration of specific ions for voltage-sensitive and

ion-sensitive channels, respectively. Propagation of this carefully orchestrated chemical and charge redistribution is utilized by neurons to send activating signals through the axon (action potentials) to induce neurotransmitter release at the synapse (Hodgkin, 1951).

In the postsynaptic cell, sodium is often the first molecule to initiate signal responses during transmitter activation at excitatory synapses. AMPA receptors clustered at the synapse surface are fast acting channels that are permeable to sodium upon presynaptic glutamate release (**Figure 1.3**) (Dingledine et al., 1999). Fast AMPA-dependent sodium currents reduce the voltage difference (depolarize) across the membrane, enough to initiate the voltage-gated ion currents such as sodium currents through NMDA channels.

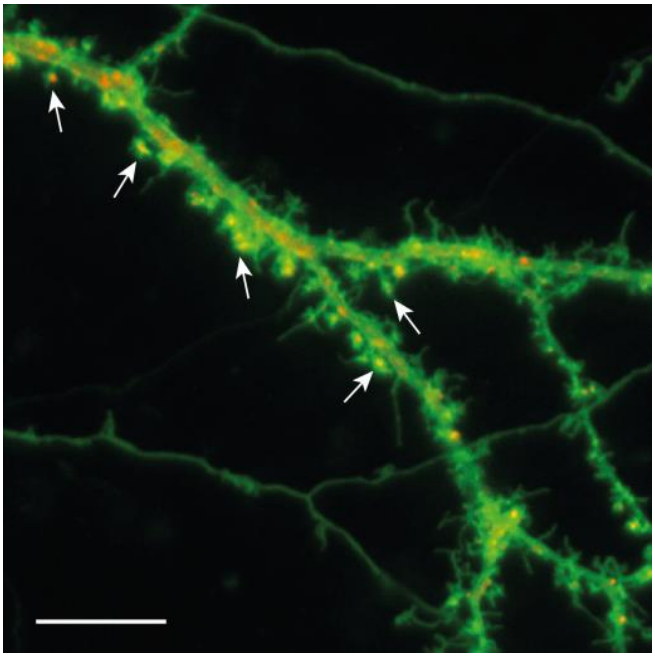

Figure 1.3 Fluorescent expression of AMPA receptors (GluR2 subunit) in cultured hippocampal neurons. Micrograph shows green and red fluorescence from a membrane ion pump and GluR2, respectively. Arrows indicate some spines containing AMPA receptor clusters. Scale bar = 10 µm.

When the temporal summation of passively diffusing dendritic depolarization reaches a threshold level at a voltage-sensitive site close to the start of the axon, an action potential is initiated. This site is called the axon hillock, and the process of chemical to potential transduction from synapse to axon hillock via the dendrites is the primary function of neurons. Their purpose is to generate regulated action potentials, triggering release of neurotransmitters for further action potential generation or ultimately for peripheral stimulation of other cell types such as muscle fibers or other innervated tissue. In the excitatory glutamatergic signal cascade, sodium is an essential activity initiator. When channel activation subsides, the sodium baseline is slowly reset by an active process. The responsibility of maintaining the sodium baseline and, thereby the resting membrane potential, is assigned to the sodium-potassium adenosine triphosphatase.

1.3.1 The sodium potassium pump

The fundamental importance of the sodium-potassium adenosine triphosphatase (Na,-K-ATPase, sodium pump) that is present in every animal cell type is evident in the Nobel Prize given to Jens Christian Skou in 1997 for its discovery. The sodium and potassium balance governed by the pump consequently controls cell volume by maintaining proper osmolarity. Ion transfer occurs as one ATP molecule is hydrolyzed to induce a conformation cycle of the pump that transports 2 potassium ions into the cell and 3 sodium ions out of the cell (Skou, 1965, Skou and Esmann, 1992). Because this process is electrogenic, or charge generating, the potential across the membrane decreases (larger negative value) as more net positive charge is moved out of the cell. This role of continual maintenance of sodium and potassium balance has given the pump the nickname of "housekeeper" protein.

The sodium-potassium pump consists of multiple subunits (Blanco, 2005). It is still unclear how necessary all subunits are for function, but it is generally assumed that functional complexes of the subunits exist as a dimer or trimer. The alpha subunit is the major enzymatic unit of the pump which exists in 4 isoforms. Each isoform has a similar structure of 10 membrane-spanning helices and 3 intracellular functional domains (**Figure 1.4**) (Morth et al., 2007). Alpha 1 (ATP1a1) exists in essentially all eukaryotic cell types. The alpha 2 subunit (ATP1a2) is expressed more selectively in places like skeletal muscle and glial cells in the CNS. The alpha 3 subunit (ATP1a3) has a further specialized expression as it is found primarily in neurons (Sweadner, 1979). The most locally expressed pump isoform, ATP1a4, is only expressed in testes. Each isoform has different sodium conductance which is probably important for region-specific function (Zahler et al., 1997, Blanco, 2005). Most notably, the neuron-specific ATP1a3 has a much higher association constant for sodium than the other isoforms. This isoform has likely evolved a low sodium affinity to effectively regulate the dramatic local sodium changes that can occur in neurons during high activity.

The beta subunit is a smaller molecule with a single transmembrane helix and a large, often highly glycosylated extracellular domain (**Figure 1.4**). The main role of the beta subunit has been presented as a trafficking chaperone, delivering the enzymatic alpha subunit to the membrane from the ER (Tokhtaeva et al., 2009). Beta subunits also regulate pump expression by preventing alpha subunit degradation, and variations are also thought to influence the efficiency of the pump (Geering, 2001). They exist in 4 variants (ATP1b1-ATP1b4), but the detailed anatomical expression and function in the brain is not especially clear.

The third subunit (gamma), also called FXYD, is smaller still and probably less essential than the beta subunit (**Figure 1.4**). It exists in many isoforms that are thought to influence pump conformation and interactions with surrounding proteins (Geering, 2005). This subunit will not be discussed in any detail in this thesis.

The simplistic view of the sodium-potassium pump as a 'housekeeper" lingers within much of the neuroscience community since it has received little attention, and details of function and regulation have not sufficiently been examined. Though overly neglected, the importance of the pump is evident in its necessity. Attempts to knock-out any of

the alpha isoforms have proven lethal, verifying the essential functions in the developing organism (Moseley et al., 2007). The alpha 3 isoform has received some attention in the past few years with the discovery of its involvement in a multiple rare diseases. Both rapid-onset dystonia parkinsonism (RDP) (de Carvalho Aguiar et al., 2004) and alternating hemiplegia of childhood (AHC) (Heinzen et al., 2012) are attributed to mutations in ATP1a3 that have horrible impacts on the affected individual. AHC can even lead to mental retardation in extreme cases. Interestingly, both diseases can arise from point mutations at various locations throughout the ATP1a3 structure. This suggests not only the essential function for maintaining normal neuronal signaling, but it also implies that the pump is highly sensitive to disruption of the many phases of the pumping cycle. As mutations can impact protein conformation, there is a possibility of disrupted protein interaction that may contribute to pump malfunction or transport regulation.

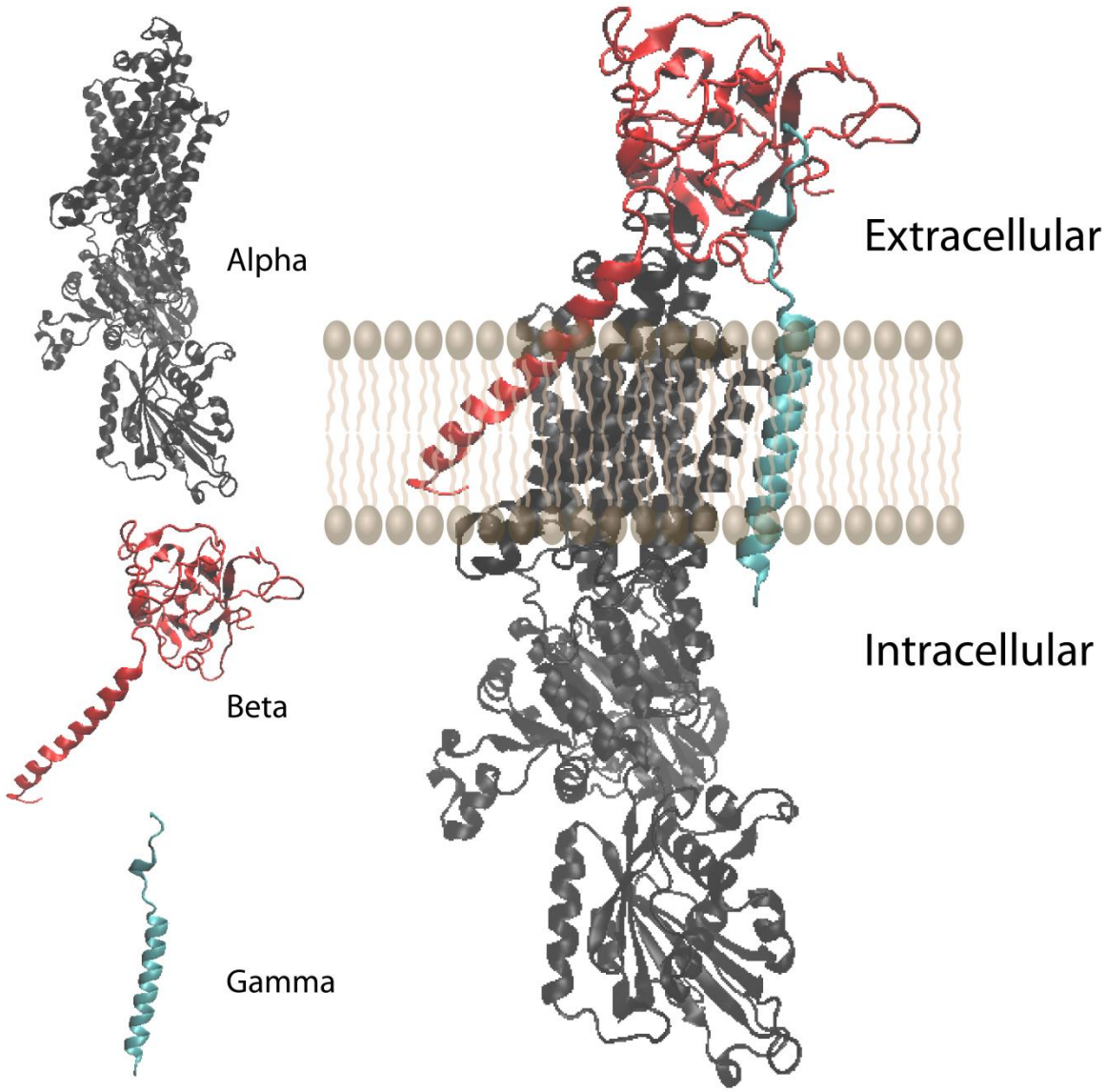

Figure 1.4 High resolution crystal structure of the Na,K-ATPase. Each subunit is shown independently (alpha - ATP1a1, beta - ATP1b1, gamma - fxyd10) and in an oligomer complex oriented within the plasma membrane. Images were constructed with Visual Molecular Dynamics (Humphrey et al., 1996), based on published structural data (Ogawa et al., 2009).

1.4 PROTEIN TRAFFICKING

Delivery of newly synthesized proteins to their respective sites of action is of great interest for both basic science and for medical or clinical perspectives. The translocation of a given protein is one of the fundamental means of regulating a proteins function. Simply put, if the protein is not in a given location, it can't be active there. All cells have a means of regulating protein abundance and transport to functional sites. Delivery of neuronal pre and postsynaptic membrane proteins is of specific interest for the medical community and pharmacological industry. We are beginning to understand the impact these environmental sensors and regulatory proteins have on the neuropsychological state of a human being, but we don't have a full grasp of transport mechanisms or how these processes may lead to new approaches for therapeutic intervention.

Nascent postsynaptic membrane proteins can be transported via a number of trafficking pathways in the neuron. We can describe 4 basic types of transport mechanisms that are most often discussed in literature: 1) Synthesis in the cell body, exocytosis in the cell body, and membrane diffusion into the dendrites. 2) Synthesis in the cell body, vesicle transport into the dendrites, and exocytosis to the dendritic membrane. 3) Partial synthesis in the cell body, transport of the immature protein into the dendrites via ER diffusion, protein maturation and membrane delivery in the dendrites. 4) Synthesis from mRNA in the dendrites, exocytosis to the dendritic membrane. These schemata are described in (**Figure 1.5**).

A majority of the membrane proteins is thought to be translated in the cell body. This is supported by the typically high abundance of proteins found in the cell body in immunolabeling or expression studies. Taking this into consideration, it is assumed that most delivery of mature, newly synthesized protein to dendrites occurs via pathway (1) or (2). Diffusion as a means of delivery is relatively slow. It is an inefficient and indirect pathway for reaching specific sites in the distal regions of the dendrite branches. However, if the protein is required throughout the cell, there is potentially a cost benefit to using diffusion as a means of transport because it is passive in nature, requiring no chemical energy but rather relying on the inherent thermal energy in the fluidic plasma membrane. The alternate pathway used by many membrane-destined proteins is the secretory pathway in transport vesicles. In contrast to the indirect lateral diffusion, vesicle trafficking is a rapid and direct pathway for delivery of protein cargo to the most distant compartment of the cell. For example, this is a practical means of transport for proteins required at the axon tip, which can even be centimeters or more away from the cell body. It enables site specific delivery of numerous protein copies in single vesicles, providing rapid changes in protein density. But the drawback of this high degree of targeting is the energy cost. Active transport of vesicles occurs along transport tracks defined by microtubule cytoskeletal structures at the expense of ATP. If active anterograde transport is not necessary for a specific protein, it may be a major cost benefit for the cell to adopt a passive transport mechanism. This cost analysis is a likely a major evolutionary determinant of the transport pathways assigned for each membrane protein.

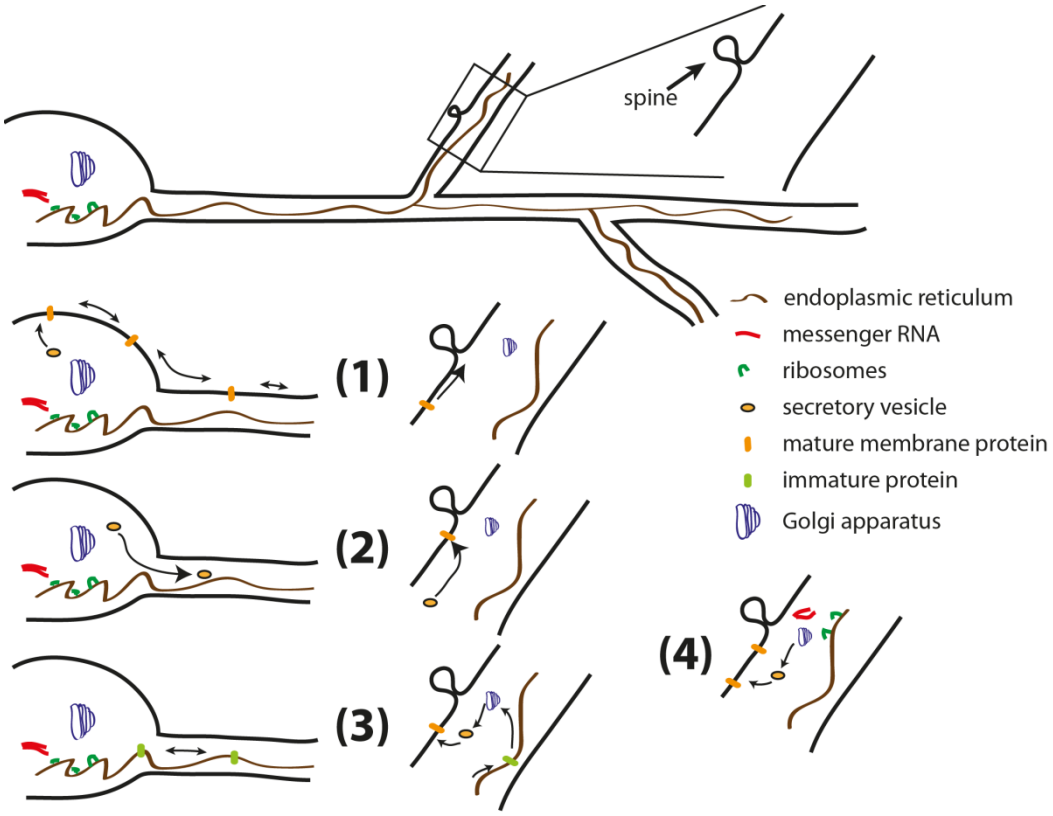

Figure 1.5 Schematic description of delivery mechanisms for postsynaptic membrane proteins. 1) Lateral diffusion in the plasma membrane. 2) Intracellular vesicle transport. 3) Diffusion in the ER. 4) Local dendritic synthesis.

1.4.1 Lateral diffusion as a synaptic regulator

Movement within the plasma membrane, often referred to as lateral diffusion, has been explored for numerous synaptic receptors over the last decade. These studies have revealed membrane diffusion at the synapse is a major determinant of synaptic strength (Choquet and Triller, 2003, Triller and Choquet, 2003, Heine et al., 2008). Controlled diffusion into and out of the synapses is a means of rapidly introducing or removing receptors from the receptive membrane region across from the presynaptic neurotransmitter release site. These studies suggest that modulation of diffusion is a consequence of changes in protein interaction. Whether interacting with extracellular molecules (Groc et al., 2007, Renner et al., 2010, Aoto et al., 2013), other membrane proteins (Scott et al., 2006, Heine, 2012),or intracellular accessory or scaffold proteins (Bats et al., 2007, Kessels et al., 2009, Liebmann et al., 2012, Opazo et al., 2012), these altered interactions change the probability of stable residence at the synapse and thereby shape the size of the receptor cluster domain and the transmission response to synaptic release. Likewise control of diffusion outside of the synapse has been described as a means of modulating the extent of membrane protein accessibility to the synapse (Groc et al., 2004, Di Biase et al., 2011).

The changes in synaptic environment described by altered diffusion regulation are often described in the context of changes in synaptic plasticity (Makino and Malinow, 2009, Bassani et al., 2013); a lasting restructuring of the synapse resulting in an amplified or diminished response to synaptic release. We are beginning to understand that these

complex restructuring processes of synapses are the underlying biophysical changes that shape learning and memory formation. Synaptic changes occur under conditions of sustained synaptic activity increases (Lissin et al., 1999, Snyder et al., 2001, Makino and Malinow, 2009, Anggono and Huganir, 2012) or silencing (Turrigiano et al., 1998, Arendt et al., 2013). Measured diffusion rates are often modulated in an activity-dependent manner. Through targeted activation of specific receptors or receptor groups, it has been shown that receptor mobility can be influenced differentially according to the activated signal pathway (Groc et al., 2004, Groc and Choquet, 2006). Most lateral diffusion studies of neuronal membrane proteins have described synaptic receptors, but little is still known of regulated diffusion of other membrane protein types.

2 AIMS

The general focus of this thesis was to provide insight into the spatial organization and dynamic transport mechanisms that govern the function of postsynaptic neurotransmitter receptors and other regulatory proteins implicated in neuropsychiatric diseases.

Specific aims of the included studies:

- Identify general and unique transport mechanisms of postsynaptic G protein-coupled receptors to facilitate targeted modulation of receptor abundance at synapses.
- Investigate the postsynaptic role of the sodium-potassium adenosine triphosphatase and define the site of action by high resolution imaging in matured hippocampal neurons.
- Examine dynamics of the neuron-specific sodium-potassium adenosine triphosphatase localization at the excitatory synapse.
- Identify a unique physiological approach to regulating the sodium-potassium adenosine triphosphatase postsynaptic transport dynamics and function in neurons.

3 METHODOLOGY

3.1 FLUORESCENT PROTEINS

A majority of the work contained in this thesis is product of imaging fluorescent proteins (FPs). The importance of fluorescent proteins has been acknowledged with a 2008 Nobel Prize in Chemistry. The original, and still essential, FP for use in biological research was extracted from jellyfish by Osamu Shimomura in the 1960's (Shimomura et al., 1962). With advances in molecular biology techniques, this green fluorescent protein (GFP) was cloned and fused to other proteins as a visual locator in *C. elegans* (Chalfie et al., 1994). More recently, GFP and other natural FP variants have been modified and expanded to resulting with a large diversity of spectral properties, much of which was championed by Dr. Roger Tsien (Tsien, 1998). With proper application, FPs have become a tremendous tool for accessing the localization and interaction mechanisms in the brain and throughout cell biology, even allowing for simultaneous investigation of multiple proteins in living cells (**Figure 3.1**).

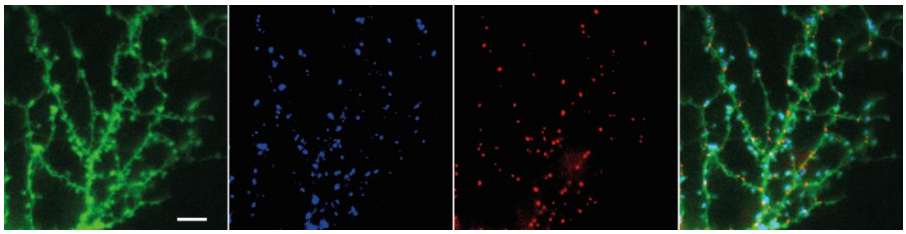

Figure 3.1 Example demonstrating multiple proteins expressed with different fluorescent protein tags in a cultured hippocampal neuron. This allows simultaneous optical investigation for interaction or localization studies within a single sample. Scale bar = 5 μm.

3.1.1 Photoactivatable fluorescent proteins

In the last decade, there has been extensive use of a new class of FPs termed photoactivatable fluorescent proteins (PAFPs). The common feature of these diverse proteins is their ability to change spectral properties upon light activation. Among them are proteins that can switch from non-fluorescent to fluorescent state, such as photoactivatable GFP (PAGFP) and Dronpa, or convert from one color to another, as seen with Kaede or EosFPs. The increasing palette of PAFPs has allowed development of the recent super-resolution imaging techniques that will be described later. As its name implies, photoactivated localization microscopy (PALM) relies on PAFPs for single molecule detection.

3.1.2 Superecliptic pHluorin

One specialized FP used frequently throughout this thesis is a GFP variant called superecliptic pHluorin (SEP). This protein is a product of GFP mutation that resulted in a highly pH-sensitive fluorescence (Miesenbock et al., 1998). It is an ideal tag for membrane protein visualization because they are normally delivered to the plasma membrane in transport secretory vesicles, which have a high acidic internal

environment. During transport, the protein domain that will ultimately reside outside of the cell plasma membrane will be subjected to the acidified vesicle lumen. If the SEP tag can be placed within this domain, the protein population in the membrane will be much more visible than the quenched intracellular population which will provide limited background fluorescence (**Figure 3.2**).

Expression of SEP-tagged proteins provides a measure of membrane abundance and allows monitoring of insertion dynamics. The quenched fluorescence of SEP in vesicles is lost as vesicles are fused to the plasma membrane and the surrounding environment pH rapids changes to that of the neutral extracellular fluid. If the abruptly increased fluorescence can be isolated by limiting background membrane fluorescence with bleaching, one can monitor exocytosis events and follow the fluorescence of the newly inserted molecules to assess trafficking regulation of nascent or recycled membrane proteins.

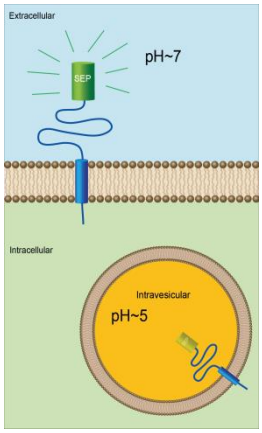

Figure 3.2 Diagram representing use of superecliptic pHluorin as a plasma membrane protein tag. Fluorescence is quenched in the acidic vesicle lumen within the cell. Once the secretory vesicle fuses with the membrane, the SEP molecule is exposed to a neutral pH environment and is no longer quenched.

3.2 CONFOCAL MICROSCOPY

The undisputed powerhouse of current biological microscopy is the confocal microscope. Extensive use of confocal microscopy in these studies warrants a brief introduction. The concept was patented more than 50 years ago as described in the more recent memoirs (Minsky, 1988) describing a method for isolation of focal plane fluorescence. Instead of collecting all excited fluorescence in a sample as is done in wide-field imaging, the pinhole in a confocal system blocks fluorescence from above and below the focal plane of interest, providing optical sectioning of a sample. Practically, this allows for axial (z dimension) resolution approaching 500nm.

3.3 FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING

FRAP, originally designated as fluorescence photobleaching recovery (FPR) (Axelrod et al., 1976a), is a useful imaging technique owing to its relative ease of application and informative output. The method is used to describe the ensemble diffusion of a fluorescent sample in a defined area or volume. It was used initially to describe membrane mobility of acetylcholine receptors (Axelrod et al., 1976b). A general experiment is conducted by eliminating fluorescence (bleaching) of a defined area or volume and monitoring the extent and rate of recovery of fluorescence. The exchange

of bleached molecules with fluorescent molecules from outside of the bleached domain is monitored with time-lapse imaging (**Figure 3.3**).

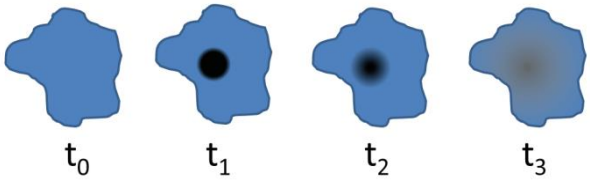

Figure 3.3 Cartoon describing steps during a FRAP experiment. T_0 represents the region prior to bleaching (initial baseline). T_1 represents frame immediately after bleaching. Acquisition usually continues until intensity in the bleached region is equilibrated.

Effective diffusion coefficients can be estimated from FRAP recovery data, but assumptions must be made for geometric parameters. For complex shapes, recovery is often rather described by a half-recovery time or half-time. This is the time required for recovery of half of the initial fluorescence. Half-time is a useful parameter to reflect differences in molecular exchange under different experimental conditions. Additionally, FRAP experiments can describe the extent of mobility or the mobile fraction of the investigated molecule (**Figure 3.4**). Incomplete recovery of fluorescence can be attributed to the inability of bleached molecules to leave the bleached area due to immobilization or confined diffusion.

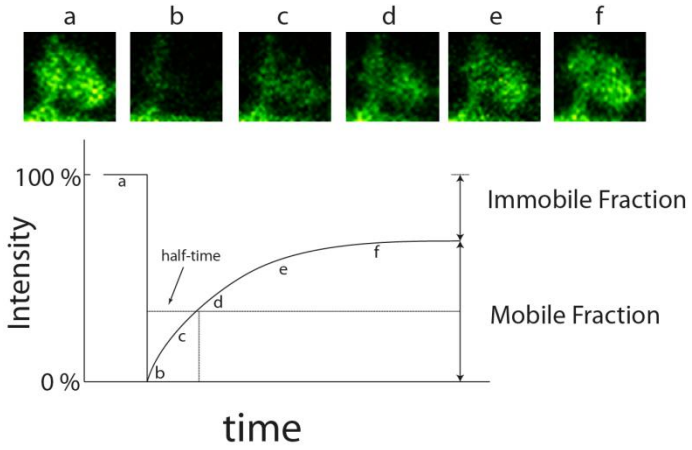

Figure 3.4 Example FRAP images of a dendritic spine and synthesized intensity profile demonstrating how half-time and mobile and immobile fractions can be extracted.

In this thesis, we have applied FRAP for comparative studies, but also to identify unique transport characteristics in neurons. Though FRAP recordings are typically used to assess membrane diffusion, we have applied bleaching recovery to examine bimodal transport in dendrites of neurons. Construction of kymographs from FRAP recordings gives a visual portrayal of both passive and active transport. Kymographs were used to verify both gradual recovery from membrane diffusion and discrete increases from actively transported clusters. Finally, FRAP was used as a tool to describe fragmentation of intracellular bodies. Without visual resolution of bodies in close proximity, FRAP can be used to identify fragmentation by limited diffusion of molecules within discrete compartments.

3.4 ANTIBODY CROSS-LINKING

Antibody-induced cross-linking or immunoprecipitation can be useful for targeted immobilization of specific membrane proteins. This technique was used on multiple occasions in this thesis. Cross-linking occurs when abundant polyvalent, polyclonal antibodies are applied to a live cell where the reactive epitopes are extracellular and diffusing in the membrane. Polyvalency allows each antibody to bind to multiple protein copies while a polyclonal antibody source amplifies the network binding by providing additional binding sites on each protein molecule. Given extracellular binding sites and enough time for the surface proteins to diffuse within proximity to each other, antibodies induce surface precipitation or clustering (**Figure 3.5**). This has been demonstrated as a practical tool in previous mobility studies (Bats et al., 2007). This method allowed us to examine the effects of targeted immobilization while maintaining an otherwise functional cell.

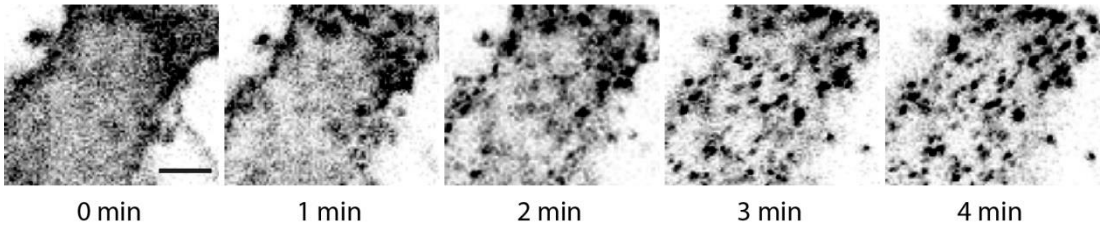

Figure 3.5 Time course of antibody cross-linking in the membrane of a living neuron. Scale bar = 2.5 µm.

An additional practical application of cross-linking is for interaction studies. As the demands for evidence of protein interaction are escalating, additional tools for measuring interactions in living cells are useful. To explore physical interactions, additional proteins can be examined for reorganization as seen with the cross-linked target. If a secondary protein is localized to the antibody-induced clusters it is likely due to either direct or indirect (through intermediate bound proteins) physical interaction.

3.5 SUPER-RESOLUTION IMAGING

From the start of optical microscopy, object resolution has been limited by the diffraction of light. Even the best light microscopes were not able to break the diffraction limit until the recent methodological breakthrough. With the advent to stimulated emission depletion (STED) microscopy, a door opened to new methods for increasing the resolving capacity of common light microscopy. To observe fluorescent molecules beyond the previous limit of about 200 nm, STED integrated the optical trick of emission depletion through a secondary quenching laser. Additional approaches were soon developed to further improve the resolving potential of the scanning or wide-field microscope. These new methods will undoubtedly play an increasing role in imaging of biological samples as most of the interactions of interest exist at the nanoscale (a single protein copy can be approximated at 10 nm). A few additional methods capable of approaching this nanoscale resolution are applied to this thesis and will be mentioned.

3.5.1 Structured illumination microscopy (SIM)

Structured illumination microscopy (SIM) is attractive as a super-resolution imaging method because of its simplicity of use. The name is attributed to the use of a grid in the light path that forms a structured pattern from the sample fluorescence. Use of a rotating grid generates a diffraction pattern that is superimposed on the fluorescent sample. After a series of images with different grid positions at a single focal plane, algorithms are applied to the patterned image sequence to extract high frequency information that is applied to the final reconstructed image. SIM can be essentially applied to any sample preparation that allows wide-field imaging. All fluorescent proteins and dyes are compatible with SIM. With a lateral resolution improvement to 100 nm, SIM cuts the fundamental diffraction limit in half. An additional highlight of a SIM microscope over most other super-resolution methods is that it provides an inherent improvement in axial resolution to below 300 nm. This is a useful and relatively easy method to apply as a user, but the resolution does not approach that of the pointillistic imaging methods described below. A few additional super-resolution methods apply pointillistic localization to discrete molecules or entire protein populations to further improve the measurement precision and thereby the image resolution.

3.5.2 Photoactivation localization microscopy (PALM)

A set of super-resolution imaging methods to arrive following STED are the pointillistic approaches called photoactivated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM). Though they are different in practice, the fundamental concepts are the same. With wide-field imaging, the diffraction-limited fluorescence from each molecule will overlap, generating a blurred image. In the case of PALM or STORM, fluorescence is limited to a sparse number of molecules that can individually be detected and localized with high precision. After detection and bleaching of the excited molecules, fluorescence is induced in a new set of molecules for detection. This sequence is repeated until sufficient numbers of molecules are imaged to generate a map of super-localized coordinates that are reconstructed into a final image (**Figure 3.6**).

PALM is used in this thesis to describe the distribution of membrane proteins. In PALM, fluorescence is limited by using a probe that is inherently dark in the emission wavelength. Specialized fluorescent proteins called photoactivatable or photoconvertible fluorescent proteins are used so keep the majority of target molecules in a dark state or in a fluorescent state differing from the acquisition color, respectively. With controlled activation from a specific light wavelength, a low number of discrete molecules can be converted to the imaged fluorescence state for localization fitting. Repetition of a few thousand acquisition frames is usually required to activate the sample sufficiently to resolve the organization structure of the tagged sample.

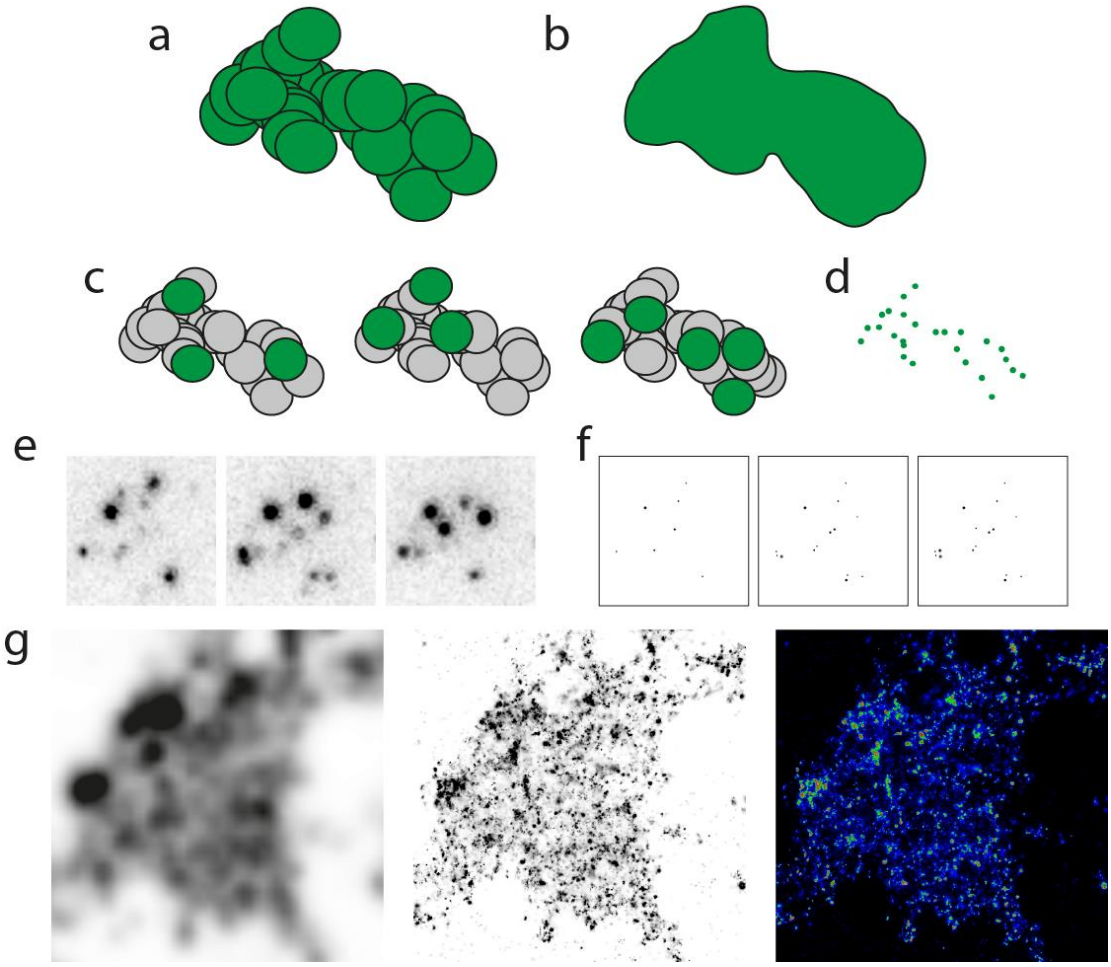

Figure 3.6 Illustration and demonstration of photoactivated localization microscopy (PALM). a) Representation of a population of labeled molecule with individual size representing diffraction-limited fluorescence. b) Wide-field diffraction-limited image acquired by a microscope. c) Sparse activation/acquisition and photobleaching for sequential frames. d) Super-resolved image reconstructed from acquisition series after pointillistic fitting. Note the filament appearance of the molecule organization that isn't apparent in the diffraction limited image (b). e) Sample 3 frames acquired from mEos3.2 photoswitchable fluorescent protein. f) Cumulative localized points from (e). g) Expanded image of 3000 frames from (e,f). Left image is projection of the wide-field image. Center and right images are corresponding super-localized representations. Frame size is 5 μm on each side.

PALM imaging often provides resolved images with a detection precision bellow 20nm, making it superior to STED (normally closer to 70 nm) and SIM resolution. PALM also has an advantage that it does not require the use of antibodies as is typically required with STED and STORM. Any protein that can be expressed as a fluorescence fusion protein can be used as a PALM sample. A limitation of PALM is that it requires expression of exogenous proteins, which can lead to overexpression localization artifacts.

3.5.3 Quantum dot single molecule localization

The use of quantum dot (QD) pointillistic imaging is not often included in the main super-resolution imaging methods, but it can be included because of it sub-diffraction localization precision. The last decade has produced extensive insight into the dynamic localization of membrane proteins by applying the latest improvements in protein

trafficking techniques. A highly productive approach was the replacement of fluorescent proteins and dyes with nanomaterials for molecular tracking. The nanocrystals used, dubbed quantum dots, have a high photon yield and photostability, so they enable dynamic, high precision localization of single molecules for long durations at high temporal frequency. QDs also exhibit spectral properties that are closely coupled to their manufactured size. Though they absorb excitation light at a wide range of wavelengths, they emit photons of specific energy which is dictated by the particle diameter. This allows users to spectrally separate quantum dot emission from the other fluorescent labels used in the experimental samples. With high spectral, temporal and spatial resolution, quantum dots are well suited for investigation of membrane protein mobility.

QDs can be synthesized in the lab from of various chemical composition and size, but most biological trafficking studies have relied on commercial sources. In the constituent studies, we have used cadmium selenium nanocrystals with an additional zinc sulfide outer layer, termed core and shell, respectively. The core crystal is an estimated 5 nm in diameter. The shell is coated with a polyethylene glycol layer to limit unspecific binding and provide a conjugation platform for reactive biomolecules. We have elected to use streptavidin-conjugated quantum dots for use with biotinylated target molecules. The macromolecular size of the functionalized QDs is measured to be 15-18 nm in diameter.

In practice, single molecule or single particle tracking (SPT) experiments require 3 developed steps. Because of the extensive use in this thesis, I will mention each of them briefly.

3.5.3.1 Quantum Dot Labeling

The first step is targeting or labeling of the protein of interest. This is typically done with antibody conjugation. Highly specific antibodies are available for many researched proteins that can be used in single particle experiments. The most basic stipulation for QD localization experiments is that the binding epitope of the target protein is extracellular. Because the cells should be kept intact and normally functioning, permeation of the cell membrane is not practical. With a specific antibody against an extracellular protein epitope, the QD conjugation can be made directly to the primary antibody or to a compatible secondary antibody. With high primary antibody specificity, direct conjugation is often preferred in order to reduce the size of the labeling complex. Addition of a second antibody further displaces the quantum dot from the actual protein of interest and introduces measurement uncertainty. In our studies, we have applied direct binding of QDs to primary antibodies via streptavidin/biotin coupling. Primary antibodies against the target protein were conjugated with biotin, which binds directly to the streptavidin coating on the QDs.

3.5.3.2 Quantum Dot Image Acquisition

Once an appropriate labeling procedure has been established for the protein of interest, the data should be collected by microscopic imaging. Though the optical system required for a quantum dot SPT experiment can be relatively simple (basic wide-field fluorescence microscope), there are a few concerns of importance that should be

mentioned. Because the ultimate aim is high localized precision, system stability is extremely important. There should be no errors associated with system drift to avoid the need to align images with fiducial markers. The acquisition frequency should be on a relevant time scale for the protein of interest. Membrane proteins are typically recorded at frequencies between 10 and 50 Hz. The faster acquisition rates equate to shorter exposure times which may require increased excitation energy. With long recording times, photo-toxicity should be of concern. As biological samples are sensitive to high energy light exposure, the intensity of and frequency of illumination should both be limited. The signal-to-noise ratio should not be increased at the expense of the sample condition. With a sufficient excitation filtration, the final component in the photon light path is the optical detector. To ensure fast acquisition while keeping low levels of photo-toxicity, a sensitive camera is required. Rather than prioritizing pixel number, the detectors for SPT systems generally invest in dynamic range. Electron multiplying charge-coupled device (EMCCD) cameras have often been used for QD SPT recordings because of their amplifying gain function and negligible readout noise.

3.5.3.3 Quantum Dot Localization

The next step is the QD detection and precision localization. By detection, I mean the analytical isolation of image intensities that are associated with an individual quantum dot. Once labeled samples are imaged, a 2-dimensional Gaussian surface fitting is performed to verify single dots and to locate their sub-diffraction position (**Figure 3.7**). Our system and samples produced a localization accuracy of 10-15 nm for quantum dots.

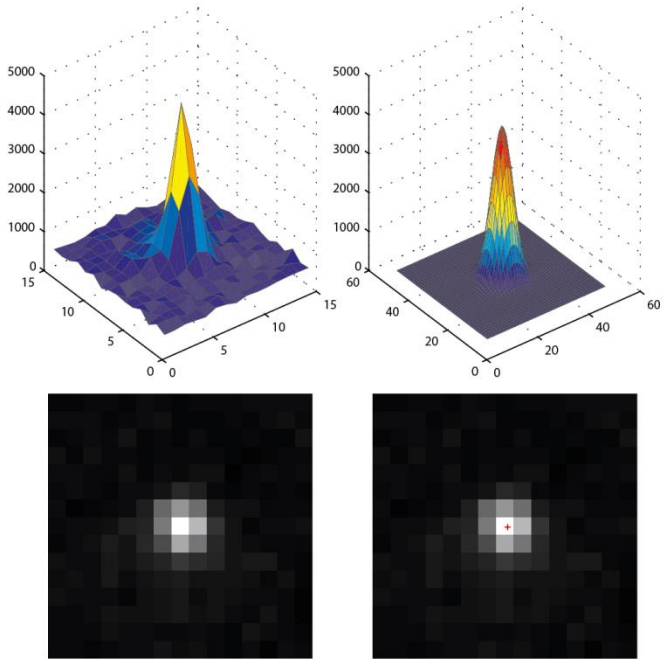

Figure 3.7 Sample fitting of a wide-field image of a single QD. QD image plotted on a surface plot. Data fitted to a Gaussian distribution for sub-diffraction localization of the true location, the fitted center point. Raw image data from the fitted QD. QD image with fitted center point marked in red. Image size is 15x15 pixels (2.4x2.4 μm^2).

3.5.3.4 Quantum Dot Single Particle Tracking (SPT)

This pointillistic, super-resolution approach can be applied with desired acquisition frequency and recording duration. After high precision locations are calculated, the 2-dimensional trajectories are made by correlation analysis of sequential frames (Bonneau et al., 2004) (**Figure 3.8**). This often requires manual validation due to loss of connection during random blinking of quantum dots.

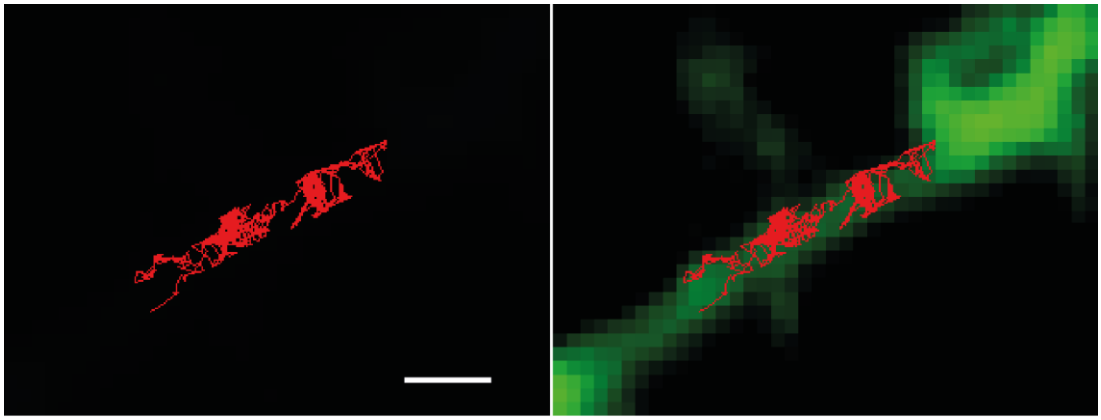

Figure 3.8 Sample trajectory of a single molecule labeled with a quantum dot. High signal and detection precision of quantum dots allows tracking at relevant spatial and time scales for membrane protein diffusion. Recording was acquired at 20 Hz. Scale bar = 1 µm.

Evaluation of neuronal membrane protein mobility often necessitates a synaptic context. The general approach to identifying synaptic regions is labeling either presynaptic or postsynaptic terminals with a fluorescent dye or locally expressed fluorescent protein. Presynaptic labeling is often done with a dye that binds to the membrane and can be internalized upon recycling of neurotransmitters via endocytic machinery after activity induction (**Figure 3.9**).

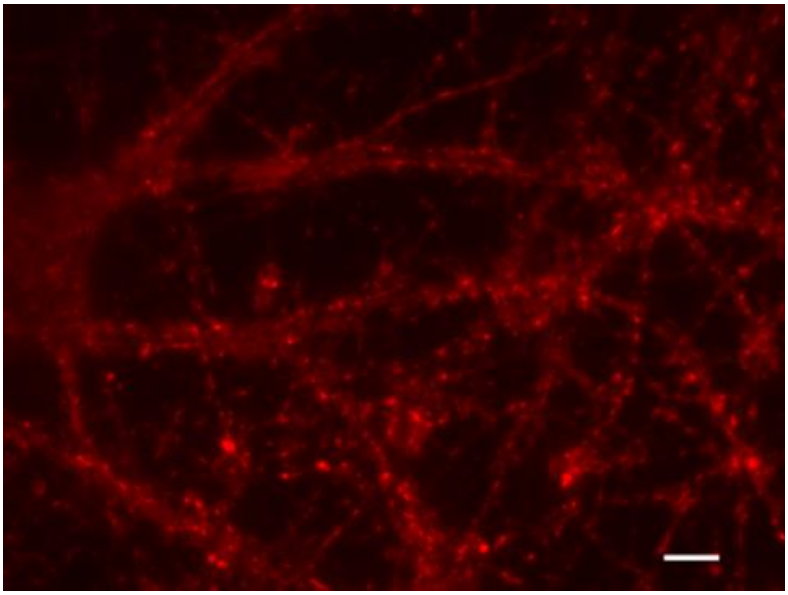

Figure 3.9. Labeling active synapses with FM4-64 dye. Scale bar = 10 µm.

In the included studies, we have used the alternative approach of labeling the postsynaptic density by fluorescent expression of synaptic scaffolding molecules that localize to the active zone of postsynaptic membranes. For inhibitory synapses, we have used fluorescent protein tagging of gephyrin. For excitatory synapses which are normally formed at spines, we have used fluorescence fusion of the scaffold molecule postsynaptic density protein 95 (PSD-95). Each protein is known to exhibit highly specific localization to the respective protein rich domain adjacent to the transmitter-receptive postsynaptic membrane. High precision localization coordinates for QDs at each acquisition frame can then be identified as synaptic or extrasynaptic (**Figure 3.10**).

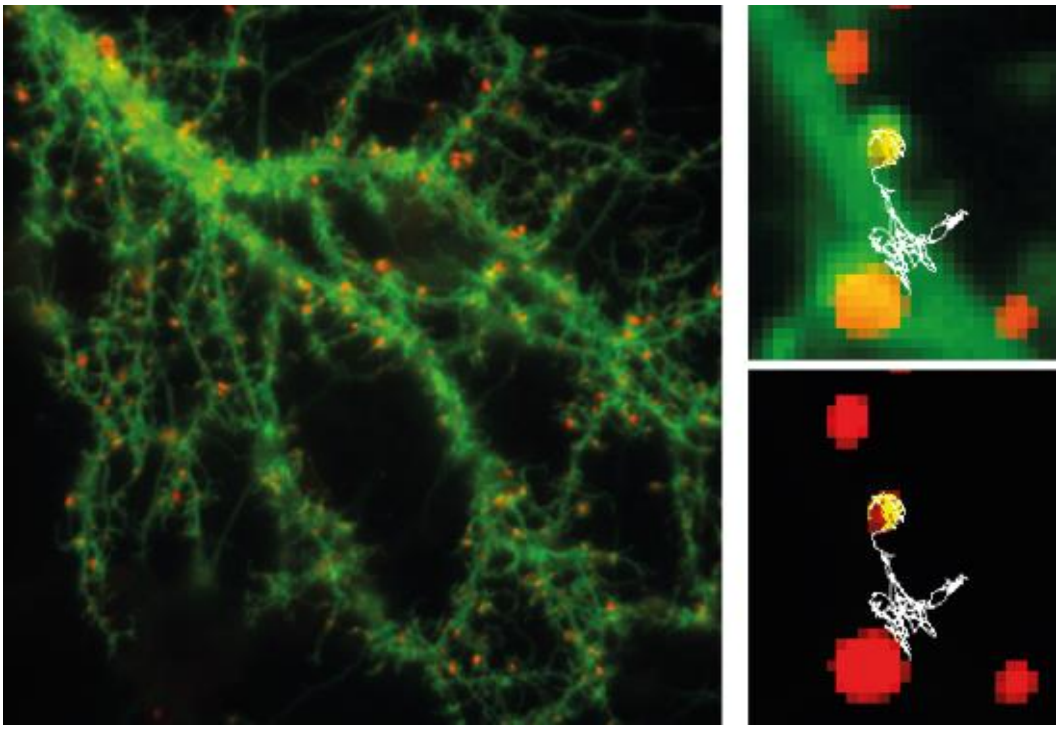

Figure 3.10 (Left) Sample labeling of excitatory synapses with expression of mCherry fused to the postsynaptic scaffold protein PSD-95. (Right) Sample 50 sec trajectory from QD showing differential trajectory coordinates between synaptic and non-synaptic membrane regions with yellow and white trajectory line colors, respectively.

3.5.3.5 Single Particle Tracking Data Representation

In addition to the descriptive trajectories extracted from quantum dot recordings, quantitative estimates can be made for comparing mobility of different molecule or differing conditions. Initially, mean square displacement (MSD) values are calculated from localization data after reconnection. The MSD of a trajectory describes the membrane area covered by single molecule within a given time interval. The relationship between area covered and time is a measure of diffusion, and diffusion coefficients are calculated directly from this relationship at short time intervals.

For the case of Brownian-like free diffusion, there is a sustained linear relationship between the mean square area and time interval. This means that the diffusion rate at long time intervals will be the same as at short intervals (initial diffusion). This

unrestricted diffusion will appear as a straight line on MSD plots. For active transport that is directed (such as movement along microtubules), the displacement area will accelerate with increasing time interval. This will be reflected in an upward deflection of the MSD curve. What is normally seen in for membrane proteins is restricted diffusion. With restricted diffusion, there are interactions that prevent the molecule from diffusing freely, so diffusion measurements made at long time intervals will appear smaller than that of the initial diffusion at short time intervals. An example of each type of MSD curve is shown below (**Figure 3.11a**).

From MSD plots of individual trajectories, one can then calculate the initial diffusion coefficient for each molecule. In our studies initial diffusion was measured within the first 5 acquisition points. Diffusion values can then be plotted in boxplots to show the variation of data. Additionally, cumulative probability plots can be used to more accurately show the entire distribution of diffusion values (**Figure 3.11b**). As the name states, these plots describe the probability of finding a molecule of a given initial diffusion coefficient.

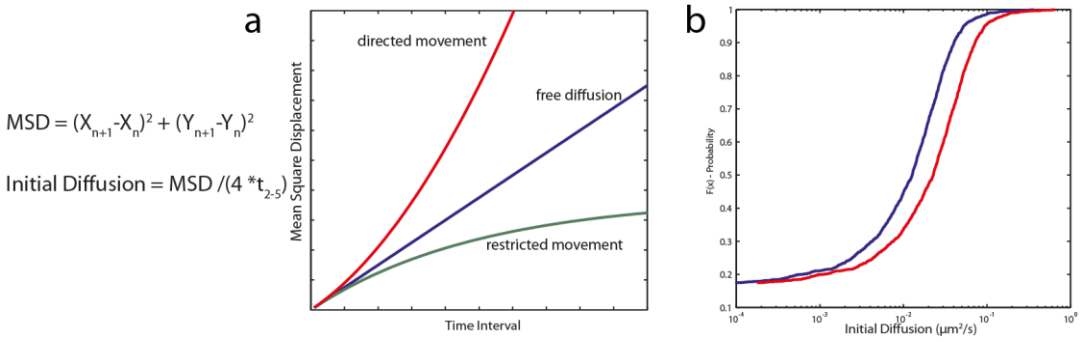

Figure 3.11 a) Simulated mean square displacement curves. b) Sample cumulative diffusion probability curves for membrane proteins.

Comparing probability curves allows detailed insight into where diffusion rates are most different between populations. In the sample above, the red curve is right-shifted in relation to the blue curve, indicating a lower probability for a given diffusion value. Reduced probability is due to the increased diffusion rate of the entire population, hence the right-shift of the curve toward the higher diffusion values.

3.6 COMMENTS ON METHODOLOGY

3.6.1 Exogenous expression

Applying GFP and other fluorescent proteins to a living system often requires introduction of foreign or exogenous DNA coding for the engineered fluorescent fusion protein. Expression of protein from plasmid DNA via transfection must be considered artificial because of the generally different mechanisms regulating expression of from plasmids as compared to the endogenous genomic DNA. In many cases, this is of little concern as expression similarity can be verified. However, as transcription promoters are not the only regulators of protein expression, exogenous expression will not always significantly alter the total protein level. Another caution is the addition of a FP to a

wild-type (WT) protein structure, as fusion of GFP can disrupt function or even translocation of the protein of interest. Most uses of fluorescent protein expression require validation of relevant expression levels, localization and function. Within each of the studies performed, exogenous and fluorescence fusion protein expression concerns are addressed.

3.6.2 Using antibodies with living cells

Conventional antibody labeling is typically performed on a fixed sample where there are no concerns of altered localization or function due antibody binding. In living cells, however, this can be a legitimate concern. As evident from the cross linking studies, an excessive abundance of antibodies can induce cross-linking of proteins and should be carefully avoided. In the SPT studies included here, high QD to antibody molar ratios were used to ensure that most antibodies have a bound crystal. In this way antibody concentrations could be titrated down to a minimal level of 0.5 ng/ml to achieve an appropriate QD labeling density typically around 25 QDs per cell.

An additional concern when using biotinylated antibodies is QD multi-valency. If there is excessive contact with many reactive primary antibodies on the membrane surface, the QDs could bind to multiple copies and induce transport artifacts through cross-linking. Maintaining the low labeling density required for isolated trajectories prevents QD multi-valency.

Antibodies, however, are inherently multi-valent. The purpose of these immunoglobulins or IgGs is to sequester foreign objects detected in the body, which is augmented by their multi-valency. This is not advantageous for trafficking experiments because it allows for the possibility of binding to two copies of the target protein, regardless of antibody density. The actual effects of valency are rather difficult to access, but there is a developing interest in producing monovalent conjugants such as fab fragments or nanobodies, both of which are monovalent components of the full antibody structures. The difficulty remains that a unique fragment must be produced for each target protein. It will be useful to ultimately combine results from IgG-based QD SPT studies with data collected from other QD labeling strategies and even fluorescent protein-based SPT methods like SPT-PALM (Nair et al., 2013).

3.6.3 Antibody limitations for precision synaptic measurements

Another concern pertaining to antibody use in the context of this thesis relates to the precision of localizing a target protein. This is a relatively new concern because it does not apply to conventional confocal microscopy where the dimension of antibodies is well below the resolution limit. However, in the expanding field of nanoscopy (microscopy at the nanometer scale), the addition of macromolecules molecules can have consequences on reported results. In this thesis we performed dynamic localization with QDs bound to the target fusion protein via a primary antibody (**Figure 3.12**). The super-resolved coordinates of the fluorescent nanocrystal are assumed to identify the location of the protein of interest, but there is an unknown degree of displacement that adds uncertainty to the measurement, despite the measurement precision of 10-15 nm. The localization accuracy can vary depending on orientation of the antibody epitope (5 nm for SEP), the antibody itself (less than 15 nm) and the QD

(18 nm). There is a maximal potential displacement equal to the sum of the component dimensions (less than 40 nm), though the average actual displacement is likely only a fraction of this. The displacement from the antibody (and fluorescent protein) in used in this study is well within the tolerance of measurement because the resolution of the synapse mask, consisting of gephyrin or PSD-95 clusters, is a diffraction limited space and generally considered an approximate marker for synaptic localization.

A final caution when applying antibodies for trafficking measurements of synaptic membrane proteins is the potential for steric hindrance. As the synaptic membrane environment is a highly dense region and the synaptic cleft can be as narrow as 15 nm, there may be a limited capacity to penetrate the synapse with membrane proteins bound to QD/protein complexes. If synaptic entry is restricted in more narrow or crowded synapses, we are likely under sampling synaptic entry frequencies. However these parameters are not reported in our study. The potential influence on trajectory information may occur at the surrounding area of the synapse, often referred to as peri-synaptic domain, but this data is incorporated into extrasynaptic trajectory measurements which will not be influenced by the nominal fraction of peri-synaptic residence time.

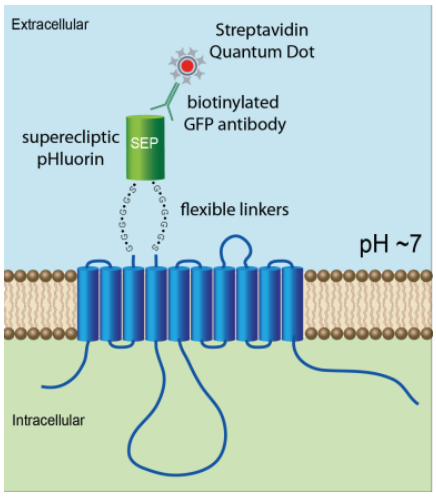

Figure 3.12 Strategy for labeling membrane proteins with quantum dots. Here is the schematic of the sodium pump, ATP1a3, expressed with an extracellular SEP insert and a streptavidin-coated quantum bound via a biotinylated anti-GFP antibody.

Steric hindrance and localization accuracy are both of some concern, and can be supplemented with various super-resolved imaging methods. As discussed previously, PALM relies on fluorescent protein expression without the need of antibodies or additional dyes or QDs. With PALM imaging there is often an option to use intracellular fluorescent tags that aren't limited by the narrow dimensions of the synaptic cleft. The included studies of the sodium pump utilize PAFPs at the N-terminal end of each pump as a comparison with the extracellular SEP expression described above. Ideally, these two approaches can be combined to perform what is called PALM SPT with living cells. This new method uses the high precision of pointillistic imaging in combination with antibody-free labeling. It is not yet clear how comparable these experimental results are, but it will be important for future validation and motivation for methodology refinement.

4 SUMMARY AND DISCUSSION

4.1 POSTSYNAPTIC GPCR RRAFFICKING

The importance of GPCRs as environmental sensors in the brain was described in the background section of this thesis. GPCRs are responsible for a majority of the treated neuropsychiatric diseases and various other complications throughout the body. We have placed emphasis on the GPCR transport regulation to highlight the current limitations of treating diseases associated with GPCR signaling. To explain the reasoning behind examination of postsynaptic trafficking and the implications of the observed results, I will describe the underlying motivation for this study in the context of the serotonin receptors.

As mentioned earlier, serotonin receptors are a major therapeutic target for treating disorders such as anxiety and depression. They are, however, indirectly targeted. The commonly used drugs for depressive disorders are aimed at elevating neurotransmitter abundance in the synaptic cleft. Compared to untreated serotonin levels, the inhibition of serotonin uptake after release with SSRIs or tricyclics (or a reduced degradation with monoamine oxidase inhibitors) will result in higher serotonin levels upon synaptic release (**Figure 4.1a,b**). The result is enhanced signal transduction strength, as indicated with the colored arrows. An obvious problem arises when there is a multitude of different receptors responding to the same neurotransmitter elevation. In the case of serotonin receptors, there are potentially 14 different receptors both pre and postsynaptic that will respond according to the therapeutically altered serotonin level. Subsequently, each of the signal cascades will be amplified in an uncontrolled fashion (**Figure 4.1c**), resulting in an uncontrolled and often undesirable response as experienced by the individual. This lack of specificity is one potential source of the severe and abundant side effects of taking antidepressants. An alternative to this approach would be to directly target the postsynaptic receptor abundance to modulate the serotonergic signal output in a receptor-specific manner, thereby limiting side effects from undesirable signaling changes (**Figure 4.1d**). This concept can apply to the many GPCR groups that are expressed in differing subtypes throughout the brain. Far too little is known of the mechanisms regulating postsynaptic GPCR trafficking, so this thesis aims to shed light on the common and unique GPCR transport routes as a means of targeted regulation.

In the included GPCR study (Liebmann et al., 2012), our aim was to identify general and unique mechanisms of delivery to the dendritic membrane. Some evidence describes local dendritic protein production, including postsynaptic receptors (Anggono and Huganir, 2012), but there is little evidence for local synthesis of GPCRs. From typical immunolabeling studies, we observe that the GPCRs we have examined are highly abundant in the cell body, suggesting the origin of synthesis. As a bulk of protein synthesis appears to occur in the cell body, translocation is essential for delivery to the postsynaptic sites of action.

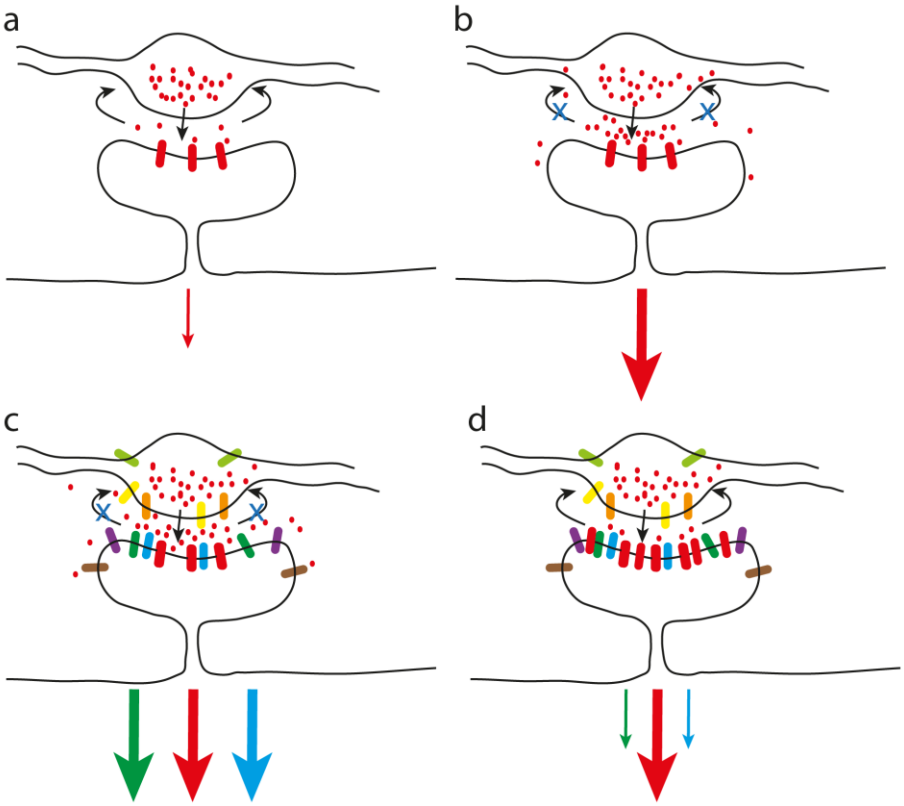

Figure 4.1 Schematic of SSRI effects on postsynaptic serotonin signaling. a) Untreated serotonin release. b) Increased serotonin in the synaptic cleft after reuptake inhibition. c) Unspecific signal enhancement from elevated serotonin exposure. d) Selective enhancement by modulation of postsynaptic receptor abundance.

Many examples in literature emphasize the role of diffusion as a delivery mechanism for neuronal receptors (Choquet and Triller, 2003, Heine, 2012). Diffusion is a passive and therefore inherently energy efficient means of transport. In this study we have examined the extent of passive membrane diffusion for the selected GPCRs. We first screened dendritic FRAP recordings by presenting recovery images with kymographs. Though this is not the conventional presentation of FRAP data, it is an ideal approach for comparing differences in transport dynamics beyond merely recovery rates and mobile pool. Kymographs reveal zones of confinement or enrichment, and they clearly distinguish between slow bulk membrane diffusion and directed transport of intracellular vesicles (**Figure 4.2**).

With exception of the 5-HT_{1b} receptor, each of the examined GPCRs exhibited a gradual recovery expected of molecules diffusing in the membrane. The unique transport pattern of 5-HT_{1b} receptors was attributed to the active transport of intracellular aggregates that were likely delivered to dendrites from the cell body. In an attempt to describe transport of 5-HT_{1b} aggregates from their somatic origin, we turned to another specialized class of fluorescent proteins. Instead of the pH-sensitivity found with SEP, we made use of the photosensitive properties of photoactivatable fluorescent proteins (PAFPs). The sequence for PAGFP was fused to that of 5-HT_{1b} and expressed in culture for 2 days to facilitate selective fluorescence within a small subpopulation of 5-HT_{1b} molecules. By limiting photoactivation to a small region in the densely

populated cell body, we could monitor the transport of the main source of 5-HT_{1b} receptors. After photoactivation, we detected directed transport of distinct 5-HT_{1b} clusters from the cell body to the dendrite branches. The distribution of exogenous, fluorescent 5-HT_{1B} clusters was undistinguishable from the results of endogenous 5-HT_{1b} immunoreactivity (**Figure 4.3**).

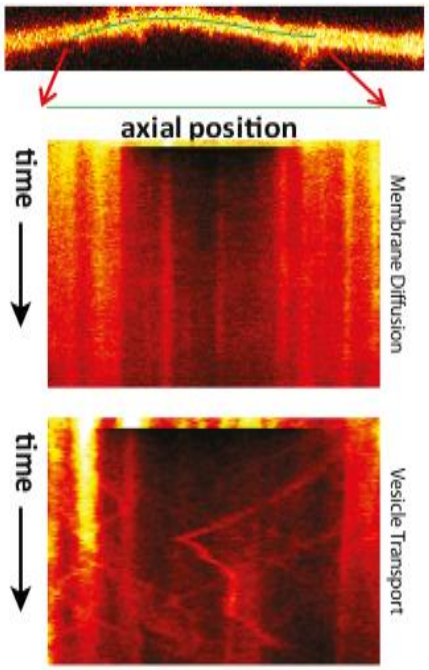

Figure 4.2 Description of kymograph portrayal of FRAP recordings. A line selected within the dendrite branch (axial position) represents the horizontal axis. The kymograph is generated by presenting this fixed region over time, increasing from top to bottom.

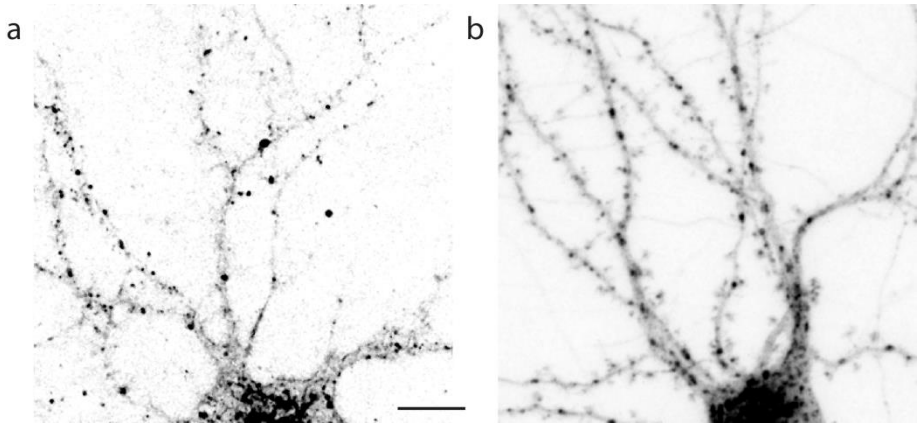

Figure 4.3 a) Immunoreactivity of endogenous 5-HT1b receptors. b) Exogenous expression 5-HT1b receptors fused to GFP. Scale bar = 10 µm.

The delivery of 5-HT_{1b} receptors can be described as active transport in secretory vesicles. Active transport is evident in the directed movement and consistent velocity of individual vesicles, whereas association with Rab8a was suggestive of secretory vesicles. To verify plasma membrane delivery, exocytosis was measured in real-time by fusing pH-sensitive SEP to an extracellular domain of 5-HT_{1b} and monitoring plasma membrane incorporation as abrupt fluorescence increases at the cell surface.

This strategy resulted in recruitment rates of similar order of magnitude as other postsynaptic receptor exocytosis studies (Petrini et al., 2009). One difficulty in exocytosis experiments is to identify the origin of individual membrane destined vesicles. In addition to the possibility of local synthesis, there is a probable contribution from recycling receptors. Regulation of membrane proteins typically includes internalization for degradation as well as recycling. We expect there is ongoing recycling of membrane 5-HT_{1b} receptors, though recycling does not seem to be a prominent source of the membrane population as seen in the low correlation with recycling vesicle markers.

The benefits of the unique 5-HT_{1b} receptor transport pathway are not directly apparent. The use of lateral diffusion as a general mechanism of GPCR delivery can be understood in the context of energy conservation. As each of the examined receptors is widely required throughout the dendritic branches, the cost of active transport for directed delivery to all regions of the neuron would likely demand too much chemical energy (ATP). The demands for the 5-HT_{1b} receptor possibly differ enough from the other GPCRs to warrant an alternative transport pathway. If overabundant 5-HT_{1b} receptors are detrimental to the function or survival of the neuron, it would be highly beneficial to retain them in intracellular stores in a releasable form for targeted delivery. This would allow controlled delivery to specific sites in the membrane when demands are high enough. This is consistent with the preferential recruitment that was detected at locations of elevated exocytosis frequency. Evidence for a need to maintain low levels of receptor abundance can be inferred in the toxicity of long-term exogenous expression. During the 48 hr limited expression used in this study, the expression pattern was similar to that seen with endogenous protein. However, longer expression times of 5-7 days were detrimental to the neuron growth.

4.2 SUPER-LOCALIZATION OF THE SODIUM-POTASSIUM PUMP

Clarification of the subcellular distribution of sodium pump isoforms has been a major challenge, mostly owing to the effectiveness of antibodies. The available antibodies that have proven useful in immunofluorescence studies all require harsh sample treatment for protein denaturing and exposure of the recognized binding epitope. Contributing to the difficulty of visually isolating the postsynaptic population of sodium pumps are the high expression levels in the postsynaptic membrane which tend to convolute the dendrite branch labeling. Efforts are ongoing to improve labeling methods, but our understanding of pump distribution and function in neurons has been constrained to a more diffuse context. We can identify the pump presence throughout the dendrites (**Figure 4.4**), but discrimination between dendritic and synaptic localization has proven challenging. Quantification has been and continues to remain a challenging task. Previous studies in our lab have aimed to assess the distribution of ATP1a3 in striatal neurons with STED (Blom et al., 2011, Blom et al., 2012), though they are only a starting point for accurately assessing the ultrastructural distribution of the pump isoforms.

Figure 4.4 Immunolabeled endogenous ATP1a3 in cultured hippocampal neurons. Scale bar = 10 µm.

The next level of investigation was to apply another set of super-resolution tools to examine the organization of sodium pumps in the dendritic membrane. To explore the differential distribution of pumps between dendritic and spine regions, we looked to nanoscopic techniques SIM and PALM. For intermediate optical resolution we imaged both ATP1a1 and ATP1a3 with GFP tags using SIM (**Figure 4.5a-c**). The major advantage of SIM is the ease of use with most commonly applied fluorescent proteins. With nanobead-calibrated lateral and axial resolution of 100 nm and 275 nm, respectively, SIM images demonstrate a high level of membrane associated expression of both pump isoforms (**Figure 4.5b**). Projections of image stacks in dendrites also reveal regions of concentrated pump nanoclusters which were often detected in the spine head (**Figure 4.5c**). This enrichment in the spine head suggests a tendency for the pump to reside in the site where sodium influx occurs during synaptic activity.

To further increase the resolution of pump localization, we applied a nanoscopy technique that utilizes a pointillistic detection method. Though PALM has been mostly limited to simple biological samples, we performed a thorough study on mature cultured neurons to demonstrate an approach to practical use of PALM for neuronal proteins (Liebmann et al., 2013). PALM images were reconstructed to observe the extent of pump aggregation or clustering in both dendrites and spines of transfected neurons. Together with mCherry-tagged PSD-95 to label the excitatory synapses in spines, PAGFP-labeled ATP1a3 was exogenously expressed for single molecule activation and localization. In corroboration with the SIM results, the most prominent clusters of ATP1a3 were associated with spines, suggesting enrichment of pumps in the spine head (**Figure 4.6a**). Some small clusters were found in the spine neck, though the relative density appeared to be much lower.

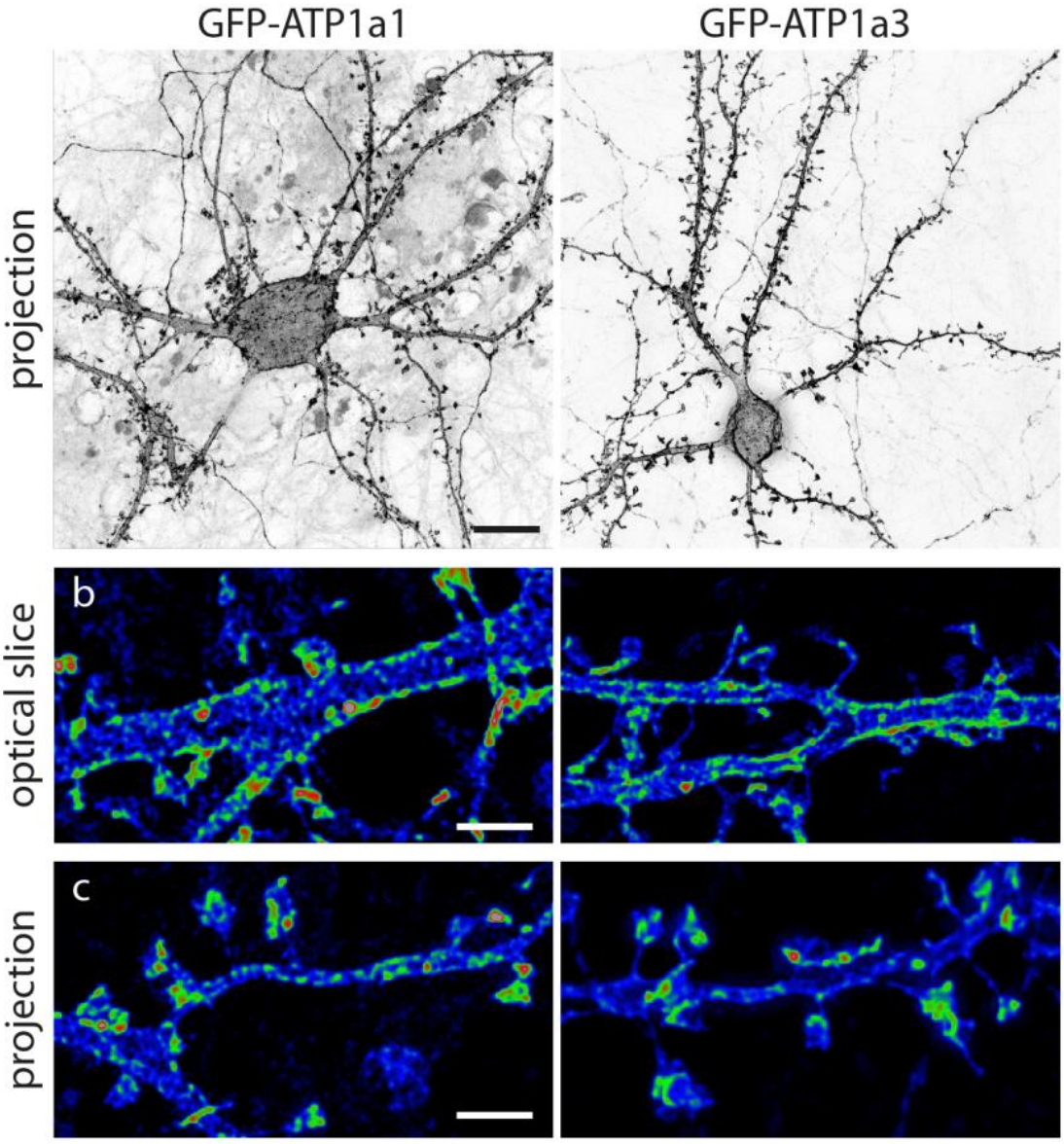

Figure 4.5 Structured Illumination Microscopy images of sodium-potassium pump isoforms in cultured hippocampal neurons. a) Overview images of ATP1a1 and ATP1a3 SIM stack projections. Scale bar = 10 µm. b) Single optical slice of the above neurons in the middle of a dendrite branch. Scale bar = 2 µm. c) Projection image of stacks from dendrite fragments. Scale bar = 2 µm.

Similar PALM results were seen from ATP1a1 and ATP1a3 with one of newest generation photoactivatable probe groups, mEosFPs, which switch from green to red upon photoactivation. mEosFPs further demonstrate that ATP1a1 and ATP1a3 are heterogeneously expressed in dendrites and form patches of high relative density (**Figure 4.6b**). Again, enrichment was seen in the spine head for both pump isoforms. This study has demonstrated that PAGFP and mEosFPs were both effective as localization probes for the sodium-potassium pumps in mature neuronal cultures. The advantages or disadvantages of the probe selection should also be considered for specific PALM applications. Because of inherent differences in photon yield, the detection precision of the probes can differ. mEosFPs have higher precision than PAGFP as seen in the provided sample histogram (**Figure 4.6c**). A drawback of the high yield mEos probes is the difficulty of bleaching which requires longer imaging times.

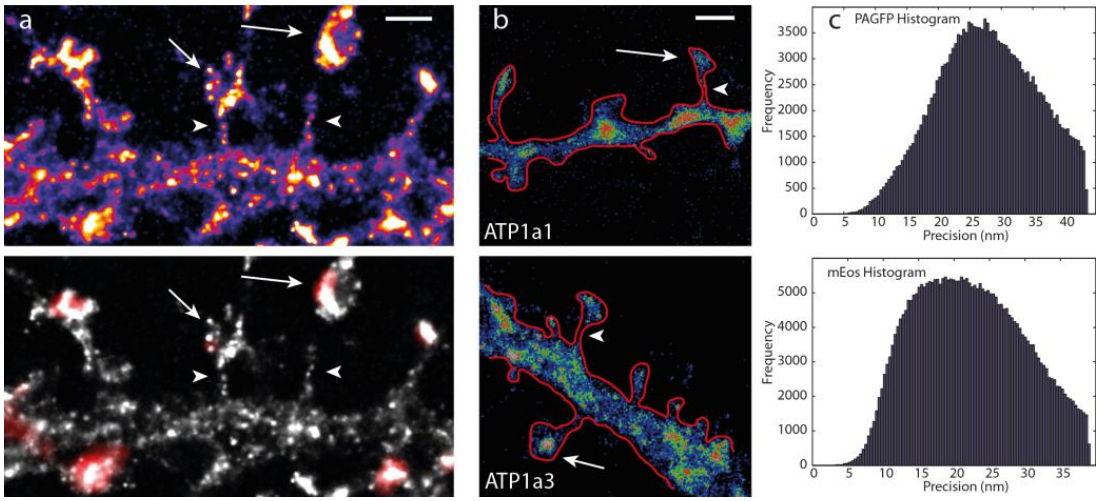

Figure 4.6 a) Reconstructed PALM images of PAGFP-ATP1a3 without (upper image) or with (gray scale, lower image) PSD-95 (red, lower image). Scale bar = 1 µm. b) PALM images with mEos2 as a photoactivatable probe. Red lines are outline of dendrite branches based on projected wide-field mEos2 images. Scale bar = 500 nm. Sample spine heads and spine necks are indicated with full arrows and arrow heads, respectively. c) Sample histograms of PAGFP and mEosFP showing average precision of unfiltered data around 25 nm and 20 nm, respectively.

A remaining challenge within the field of optical nanoscopy is the ability to accurately quantify protein densities. We have applied PALM microscopy to estimate the number of pumps in the spine head. By counting the individual activation events within the confines of the spine head, this pointillistic method allows for direct measurement of single molecules (**Figure 4.7a**). By activating every molecule expressed in the spine, the total number of present molecules can theoretically be measured. We have estimated the number of ATP1a1 and ATP1a3 molecules in spines with both PAGFP and mEosFP labeling. A major difficulty, as described for the higher precision probes, including mEosFPs, is the potential for repeated blinking which can produce erroneous quantification. Methods have however been established to correct for the extent of blinking on an experimental basis (Annibale et al., 2011). After correcting for repeated blinking, we approximate an overestimate of 33% due to repeated blinking. We find no significant difference between the two probes and a similar expression level for each of the two pump isoforms (**Figure 4.7b**). Median ATP1a1 and ATP1a3 densities were finally estimated between 450 and 650 molecules/µm^2 projected spine area.

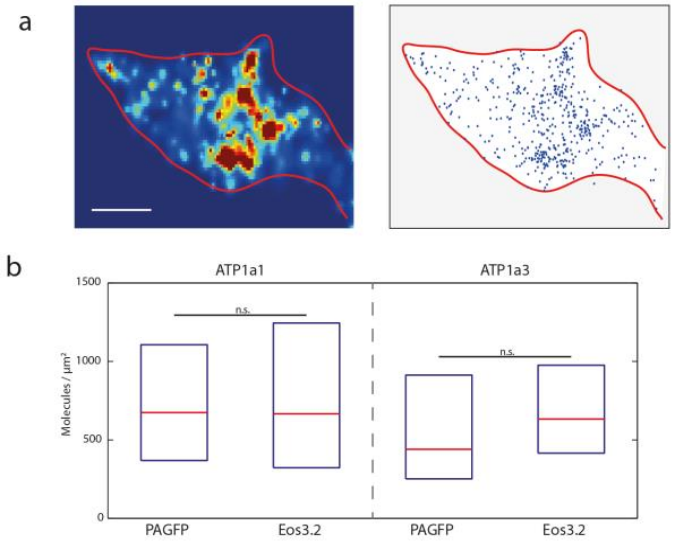

Figure 4.7 a) Single spine from a PALM image of mEos-labeled ATP1a3. On the left is a generated PALM image and right is a map of center point of each detected molecule. Scale bar = 200 nm. b) Quantification of molecular density of pump isoforms in single spine projected area after correcting for estimated photoblinking.

In addition to the high counting attributed to blinking, another concern should be addressed; the excess of molecules due to exogenous expression. Because PALM required expression of fusion proteins, it normally requires transfection of cells with plasmid DNA, resulting in an artificial promotion of protein expression. There are basic approaches to overcoming this limitation which include specific promoters for the protein of interest and generation of genetically modified mice for genomic incorporation of the fusion protein sequence. These are normally excessively expensive to justify for individual proteins, so they are generally not options. In the case of the sodium pumps, we don't believe there is a high degree of overexpression because of the fundamental limitation on expression. The number of pumps in the plasma membrane is limited by expression of the beta subunit, which is required for pump translocation from the ER to the plasma membrane (Tokhtaeva et al., 2009). Because we are not co-transfecting with beta subunit plasmid DNA, we are likely not altering the total abundance of the beta subunit, which ultimately limits the total membrane incorporation. There is however a possible displacement of alpha subunit isoforms when one is exogenously expressed. It appears that ATP1a3 can notably reduce the endogenous levels of ATP1a1 in some cells, implying that the count for each individual isoform may reflect the upper limit of total alpha isoforms in a given cell environment. Further estimates with antibody based methods such at STORM will facilitate more discussion on the accuracy of quantification attempts.

4.3 DYNAMICS OF SODIUM-POTASSIUM PUMP LOCALIZATION

4.3.1 Lateral diffusion in the postsynaptic membrane

The notion of lateral diffusion for membrane proteins is by no means an unexplored concept. In the last 10 years, we have learned a great deal of the mobile characteristics of numerous postsynaptic receptor proteins in the neuronal membrane. Yet, little is known of the role of mobility for other classes of proteins. Only a few non-receptor channels have been examined and the understanding is still relatively limited. In the second study included in this thesis, we address the question of dynamic postsynaptic localization, or mobility, of the sodium-potassium pump. The emphasis has been placed on the high capacity ATP1a3 isoform (Blanco, 2005) because of the required postsynaptic management of high sodium influx during elevated synaptic activity (Rose, 2002).

Though some may have expected a static distribution of the pump as an efficient means of regulating intracellular sodium concentrations, an alternate approach has been adopted by many cell types. Lateral diffusion of the sodium-potassium pump in cell membranes was introduced many decades ago (Jesaitis and Yguerabide, 1986, Paller, 1994) but there has been no evidence for pump mobility in the neuronal membrane. We do find however that the neuronal isoforms, both ATP1a1 and ATP1a3, are highly mobile in the neuronal plasma membrane. This study describes the FRAP experiments performed on SEP-tagged ATP1a3 in different regions of the dendritic membrane of cultured neurons. We found that lateral diffusion of the pump varies between synaptic compartments in spines and the dendrite shaft. After bleaching membrane patches of equivalent projected area (roughly 1 μm^2), we noticed that, given sufficient time, the

fraction of mobile ATP1a3 molecules (mobile pool) is similar between the two areas (**Figure 4.8**). Conversely, the rate of molecule exchange greatly differed between the regions as seen in the measured half-time recovery.

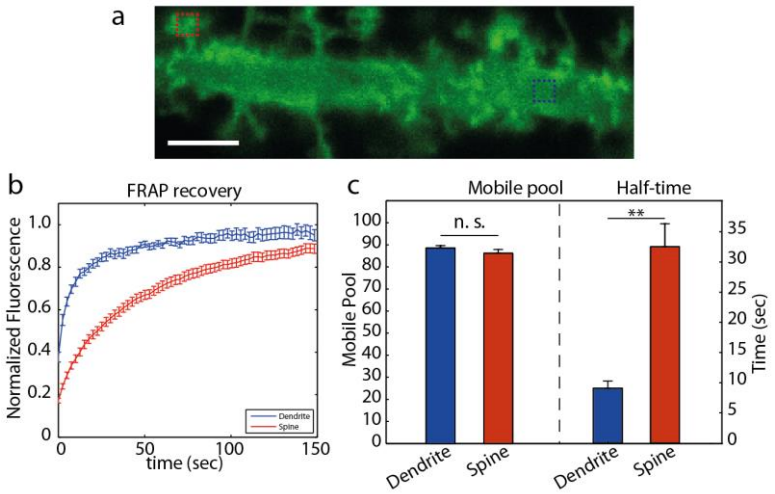

Figure 4.8 FRAP recording of ATP1a3 tagged with SEP. a) Dendrite branch with sample dendrite and spine bleach regions highlighted with blue and red boxes, respectively. Scale bar = 5 µm. b) Mean normalized fluorescence intensity profiles of indicated regions after bleaching. c) Mean mobile pool and half-time values calculated for each region.

One possible explanation for the differences in mobility is an active increase in movement of pump molecules outside of the spine compartments. The alternative is obviously that there is a restriction of pump mobility around the excitatory synapses. The latter of the two is a more appropriate description of the differences in mobility. We believe that the lateral mobility of the pump in the dendritic membrane should be described as less restricted than within the spine. The source of movement is principally the inherent thermal motion of the fluidic plasma membrane, and each pump molecule will move randomly within the confines of the membrane until restrictions are applied to inhibit motion. Restricted movement in synapses has been described even with more freely moving lipid molecules (Renner et al., 2009), demonstrating the differences between synaptic and extrasynaptic membrane composition and viscosity. Likewise, this naturally applies to the pump molecules. Morphological effects, barriers and differences in membrane composition should influence the pump mobility in different regions of the cell. The pump is however much less mobile than the lipid molecules, probably attributed to increased interaction with reactive neighboring proteins both within the membrane and in close proximity (inside and outside of the cell). The dynamics of these unknown interactions are a focus of this study.

To investigate the subtle regulation of ATP1a3 mobility, we performed a detailed examination with QD SPT. Supporting the results of the bulk FRAP measurements, SPT recordings revealed an altered movement of single molecule trajectories in proximity of excitatory synapses. Movement appeared more unrestricted outside of synapses as is evident in QD trajectories, average MSD curves and cumulative probability distributions of diffusion coefficient values (**Figure 4.9**).

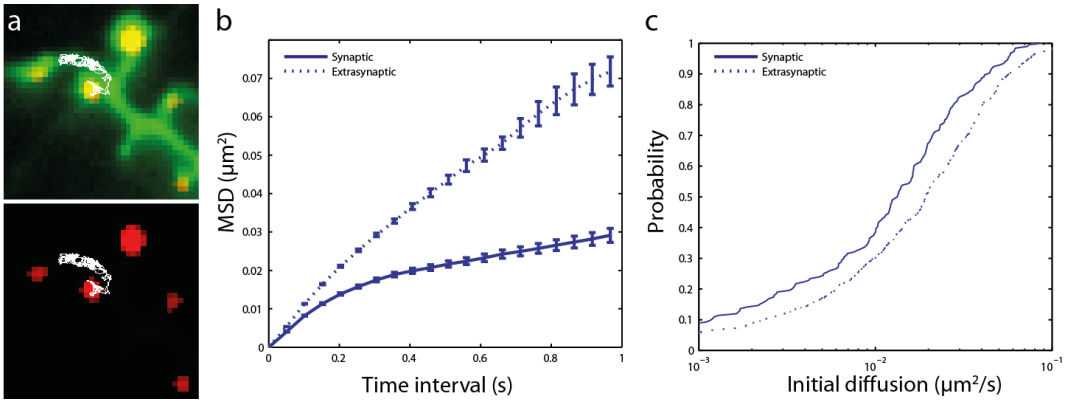

Figure 4.9 a) Sample QD trajectory of ATP1a3 in a dendrite branch. Green and red colors represent expression of SEP-tagged ATP1a3 and PSD-95-mCherry, respectively. b) Mean square displacement vs. time interval curves of ATP1a3 trajectories in the synaptic and extrasynaptic membrane. c) Cumulative probability of initial diffusion coefficients for synaptic and extrasynaptic ATP1a3 trajectories.

4.3.2 Lateral diffusion is modulated by synaptic activity

Though some regional differences should be expected for reasons described above, changes in these region-specific mobility characteristics would not only be unexpected but would also suggest interesting implications for physiological regulation of ion balance in neurons. Through a series of treatment protocols to modulate the activity at excitatory synapses, we detect a consistent and significant change in mobility within the synaptic domain and outside of the synapse (**Figure 4.10**). AMPA receptor stimulation with AMPA or glutamate resulted in an increased diffusion profile in the synapse. Blockade of synaptic activity by non-specific inhibition of neurotransmitter release with tetrodotoxin (TTx) resulted in a reduced diffusion profile. Interestingly, the stimulation of group 1 metabotropic glutamate receptors with dihydroxyphenylglycine (DHPG) also resulted in a reduced synaptic (and extrasynaptic) mobility. These results demonstrate the complexity of diffusion regulation of ATP1a3 in response to various forms of synaptic activity. It is not unusual to see altered protein diffusion responses to different forms of excitatory stimulus (Tardin et al., 2003, Groc et al., 2004), though the mechanistic changes of interactions defining the restricted mobility are not always apparent. In the case of ATP1a3 mobility, the clear activity dependence is not yet understood. Though there may be a restructuring the of membrane environment during dramatic elevation or silencing of synaptic signaling, the pronounced effect on ATP1a3 diffusion is more likely due to specific interaction, though they may be of relatively low affinity. Direct or indirect interaction with PSD-95 scaffold molecules is a potential mechanism as immunoprecipitation of the two has been reported (Zhang et al., 2009, Blom et al., 2011, Azarias et al., 2013). We also described enrichment near the PSD cluster in PALM studies above (Liebmann et al., 2013), though there is no terminal PDZ binding motif on the pump expected of the classical PSD-95 interactions (Kornau et al., 1995, Sprengel et al., 1998, Bard et al., 2010). However, this interaction does not explain modulation of mobility in the extrasynaptic domain.

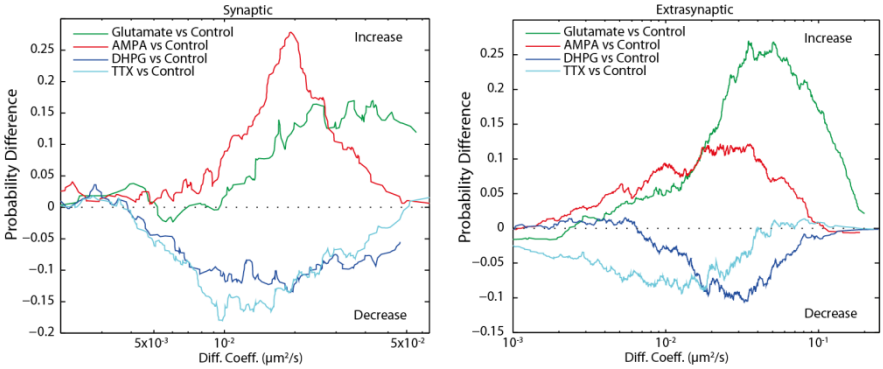

Figure 4.10 Difference plots of cumulative probability defined by subtracting treatment value cumulative probability diffusion curves from the associated control group curves. A positive value equates to a lower cumulative probability diffusion value for the treated group (see Figure 4.9c) at the respective diffusion coefficient value, meaning a faster population.

4.3.3 Lateral diffusion of the sodium pump impacts regulation of intracellular sodium

A difficult task in the elucidation of sodium-potassium pump mobility in living neurons is the evaluation of consequences on cell function. As we know the pump is responsible for removing intracellular sodium, we examined the role of ATP1a3 mobility in the capacity to clear sodium during signaling. To measure changes in sodium levels, we utilized the sodium sensitive dye Asante NaTRIUM Green-2 (ANG-2). After loading cultured neurons with ANG-2, we induced a postsynaptic sodium load by controlled stimulation of synaptic vesicle release. By applying an electrical stimulation through field electrodes (2 ms pulse, 50 Hz, 1 s burst width) we could induce a glutamate release-dependent elevation of intracellular sodium. We then correlated the sodium level decay with the rate of sodium equilibration, which is performed by the sodium-potassium pumps. To begin to dissect the role of pump mobility, here we applied the cross-linking approach previously described in the methods section. Antibody cross-linking immobilized ATP1a3 by immunoprecipitation of all membrane bound-pumps (**Figure 4.11a**). Sodium equilibration profiles reveal that cross-linking significantly reduces the rate of sodium removal as measured from the soma or dendrites of neurons transfected with ATP1a3 (**Figure 4.11b**).

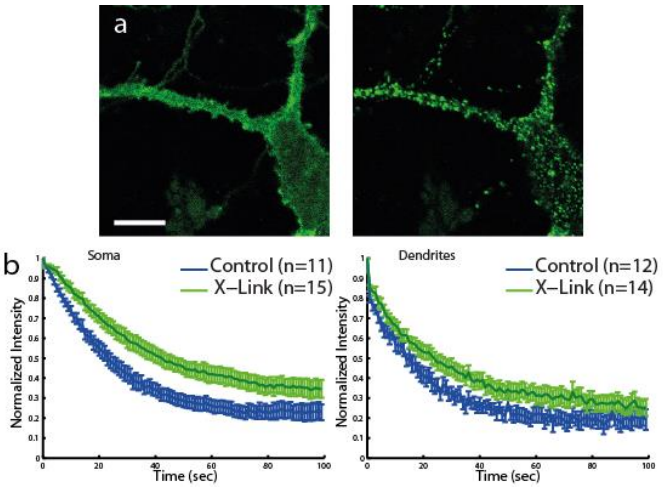

Figure 4.11 a) Images of mTurquoise2-ATP1a3 before (left) and after (right) antibody immunoprecipitation or cross-linking. b) Normalized intensity profiles of ANG-2 imaging after field stimulation of neurons transfected with mTurquoise2-ATP1a3. Individual curves are from soma or dendrite and with or without cross-linking, as indicated.

Care should also be taken not to over interpret the results from sodium imaging after immobilization. Though it is revealing at the macroscale, translation to the relevant microscale is difficult. Because we are not specifically targeting immobilization of ATP1a3 to the synapse, we are unable to resolve what the effects of mobility are within the confines of the spine. We believe that reduced diffusion should have notable effects on sodium removal of the spine simply by preventing efficient membrane coverage. This may be especially important in the spine, considering the spine neck geometry that slows ion diffusion from the spine to the dendrite branches. Ultimately, net changes in pump density in the spine are likely to have the greatest effect on sodium equilibration. If reduced mobility increases the probability of residing in the spine, there may be a positive enhancement of the sodium pumping capacity. In the context of reduced mobility seen with TTx treatment, this could suggest a feedback mechanism to prevent silenced spines from becoming overly sensitive during homeostatic up-regulation (Arendt et al., 2013).

Another basic concern that should be addressed is the effect of cross-linking on the activity of the pump. This is rather difficult to verify because repeated immunolabeling of the pump in any intact fluidic membrane will result in some cross-linking. Changing to nanobodies should avoid cross-linking, but this would likely change the binding epitope thereby making it an improper control for antibody binding effects. We will attempt to resolve the effect of antibodies in vitro, though we don't expect to see any functional effects of antibody binding. We are confident of this because the antibody binding site is far removed from the structure of the pump. Cross-linking antibodies are targeting the fluorescent protein which is inserted into the second extracellular loop of ATP1a3 and flanked by small flexible linkers. It is not likely that antibody binding to mTurquoise2 will translate a conformational change in the pump structure that will inhibit the pumping cycle.

We have started with the assumption that expression of SEP-ATP1a3 or mTurquoise2-ATP1a3 will result in functional sodium pumps. The FP has been inserted into an extracellular loop that does not interact with other pump subunits. Additionally, the proper insertion into the plasma membrane indicates undisrupted subunit hetero-oligomerization and effective translocation. We have also demonstrated, as mentioned in the previous section, that transfection of an exogenous pump isoform tends to displace a majority of the endogenous pumps. Accordingly, promotion of a poorly functional pump would destabilize sodium maintenance and rapidly kill the cell. Because SEP-ATP1a3 and mTurquoise2-ATP1a3 transfected neurons are functional and thrive for weeks after transfection, there is little reason to expect an altered pumping capacity.

4.3.4 The beta subunit of the sodium-potassium pump

The elucidation of regulated lateral diffusion of the sodium-potassium pump in postsynaptic membranes is a novelty that we expect to fuel new perspectives of sodium maintenance, but some of our ultimate goals are to clarify mechanisms of altered diffusion and specify targets for potential manipulation. In the included study, we propose the beta subunit as a promising localization regulator. As described in the

background section, the beta subunit is an important regulator of membrane translocation, but little is known of the role of beta subunits in neurons. We first propose that the beta subunit is a potential candidate for postsynaptic pump regulation by demonstrating the extent of oligomerization between ATP1a3 and ATP1b1 when expressed in cultured neurons. Through cross-linking experiments, we find that the pump remains in an alpha/beta oligomer even after transport and insertion into the plasma membrane (**Figure 4.12a**). Consequently, selective immobilization of the beta subunit will restrict lateral diffusion of the nearly all membrane-bound alpha subunits (**Figure 4.12b**).

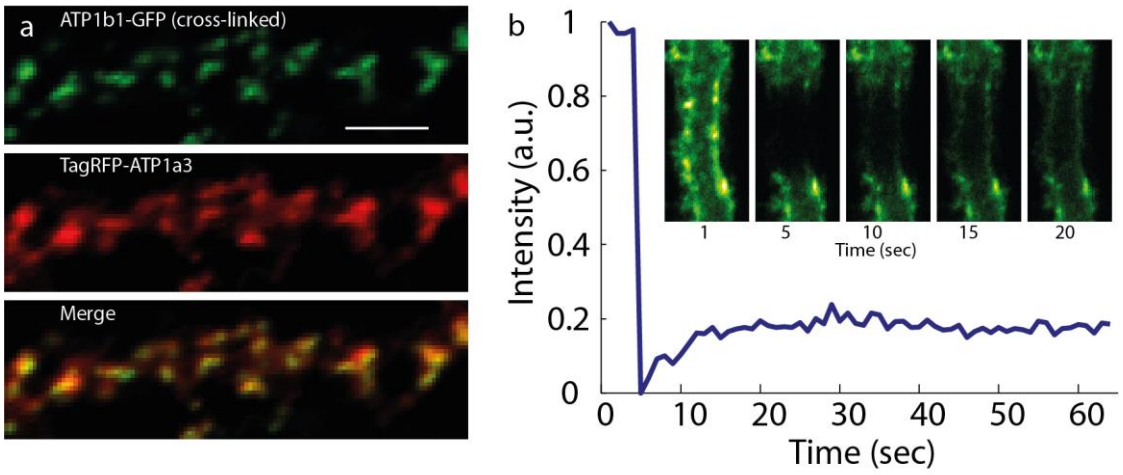

Figure 4.12 a) Confocal image of transfected ATP1a3 and ATP1b1 in a dendrite branch after cross-linking against extracellular GFP. Scale bar = 2.5 µm. b) Images and intensity curve from FRAP recording of fluorescent ATP1a3 after cross-linking of ATP1b1.

The cross-linking described above is however an artificial immobilization strategy, so we should ask if the beta subunit could be a plausible factor for pump immobilization in physiological settings. If we reexamine the structure of the sodium pump, we see that there is a large surface area of the beta subunit that is exposed for interaction in the extracellular space (**Figure 4.13**). The extensive glycosylation and large extracellular domain of the beta subunit suggest likelihood for interaction (Geering, 2001), whether specific or simply a consequence of steric hindrance in crowded extrasynaptic environments. This brings up a possibility of regulation via matrix molecules or various other components in the extracellular space. Previous studies have shown the impact of extracellular interactions for regulation of postsynaptic membrane protein mobility (Groc et al., 2007, Saglietti et al., 2007, Frischknecht et al., 2009). In kidney-derived cells, pump localization can be regulated by extracellular beta/beta subunit (intercellular) interactions (Vagin et al., 2012). Extracellular matrix molecules may contribute to diffusion regulation of the sodium-potassium pump in culture, and they would likely play an even more important role in the intact brain since matrix is highly dependent on culture conditions and development (Gundelfinger et al., 2010). An interesting proposal is the potential for trans-synaptic interaction as there are many adhesion proteins known to extend from one synaptic membrane and interact with others in the opposing membrane (Aoto et al., 2013). As we expect the typical synaptic clefts to be beyond 20 nm from membrane to membrane, trans-synaptic beta/beta interactions are not likely to routinely occur. This does not rule out the potential for interaction with other molecules which are densely packed in and around the synapse.

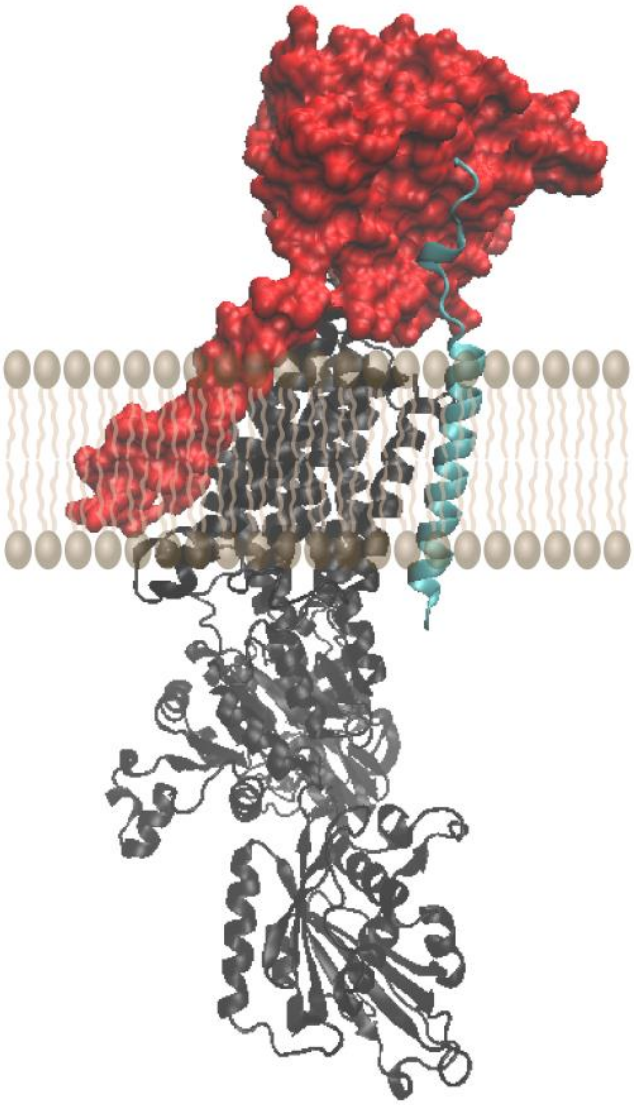

Figure 4.13 Determined crystal structures of the sodium-potassium pump subunits. The beta subunit is shown with red surface rendering to reveal the extensive surface area available for interaction in the extracellular space. Images were constructed with Visual Molecular Dynamics (Humphrey et al., 1996), based on published structural data (Ogawa et al., 2009).

4.3.5 A novel modulator of sodium-potassium pump diffusion

In the final part of the second study in this thesis, we examine the effects of a recently discovered protein with a specific and functional effect on the sodium-potassium pump. It was not evident how or where the action occurs for this novel protein, dubbed the modulator of Na,K-ATPase (MONaKA) (Mao et al., 2005). MONaKA is distributed throughout the brain and many other organs of the body. It is known to bind to extracellular motifs of the sodium-potassium pump via the beta subunit (Gorokhova et al., 2007), suggesting a secretory pathway that has not yet been identified. We generated constructs for synthesis and purification of MONaKA to examine its effects on ATP1a3 transport regulation in dendritic membranes.

After applying soluble MONaKA (sMONaKA) to cultured neurons for as little as 60 min, we detected binding and aggregation of the soluble protein along dendrites and

often in close proximity to excitatory synapses (**Figure 4.14a**). The consequence of extracellular sMONaKA interaction with the beta subunits were then assessed by monitoring the lateral diffusion characteristics of ATP1a3. Even after this short incubation time, we detected a reduced mobility of the pump in the synaptic domains (**Figure 4.14b**). Diffusion analysis revealed a pronounced reduction of diffusion coefficients within the faster domain of the synaptic trajectories (**Figure 4.14c**). Interestingly, the mobility was also reduced in the extrasynaptic membrane. This novel description of sodium-potassium pump mobility regulation suggests some interesting implications for modulating intracellular sodium, though there is still a large gap in the understanding of possible conditions and mechanisms of MONaKA secretion. The rationale for sMONaKA cluster formation around synaptic sites has not been clarified, and the physiological relevance for such aggregates should be examined.

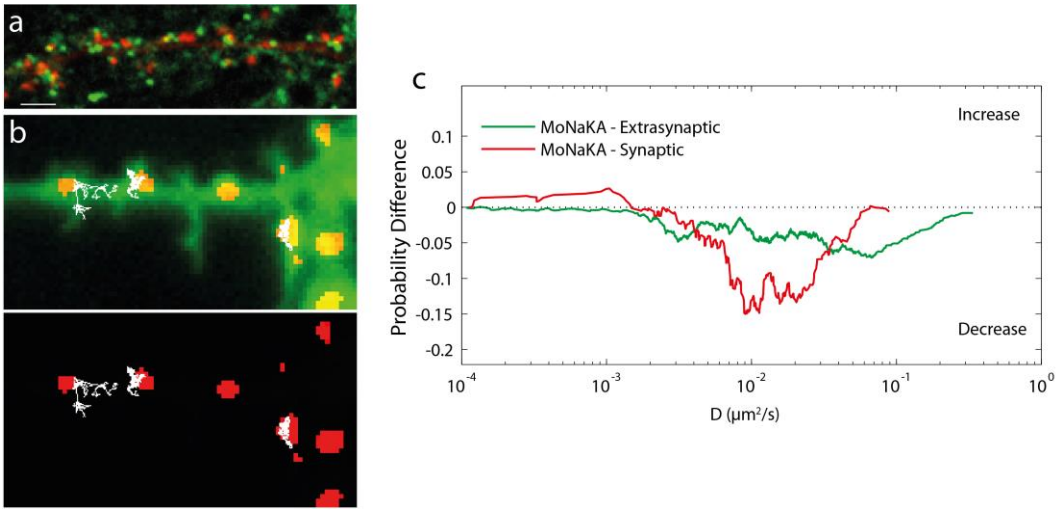

Figure 4.14 a) Confocal image of sMONaKA clusters (green) and excitatory synapses (red) of cultured neurons incubated with sMONaKA for 60 min before fixation and immunolabeling. Scale bar = 2.5 µm. b) Example of postsynaptic trajectories of ATP1a3 from QD SPT experiments after 60 min incubation with sMONaKA. c) Difference plots of cumulative diffusion coefficients for synaptic and extrasynaptic trajectories calculated as treated probability subtracted from vehicle probability at a given initial diffusion coefficient value.

5 CONCLUSIONS

5.1 POSTSYNAPTIC GPCR TRAFFICKING

The broad message of the included GPCR study is the variability of transport mechanisms for postsynaptic delivery to signaling locations. Within the small sample of receptors included in our study, we found that one deviated from the normal transport pathway. Whereas most of the examined GPCRs were transported to and throughout the dendrites via passive lateral diffusion in the plasma membrane, the 5-HT_{1b} receptor was delivered via active transport in secretory vesicles. Repeated events of 5-HT_{1b} receptor delivery were detected at multiple sites of preferential exocytosis. Once inserted into the membrane, receptors underwent diffusion until they were temporally confined at synaptic sites.

5.2 NANOSCOPIC LOCALIZATION OF THE SODIUM-POTASSIUM PUMP

Resolution of the subcellular distribution of the sodium-potassium pump has been elusive. With super-resolution optical techniques we have shed light on the nanoscale dendritic topology of the neuronal pump isoforms. We have demonstrated that ATP1a1 and ATP1a3 are arranged in nanoclusters throughout the dendrite shaft and within the spine head. Multiple imaging approaches revealed an enrichment of pumps in the spine head where sodium maintenance demands are high during synaptic activity. In addition to nanoscopic evaluation of the pump distribution, PALM imaging allowed us to quantify pump abundance in spines. With multiple probes we estimated a range of 450-650 pumps/μm^2 of projected spine area. This suggests that the pump likely plays a more prominent role in sodium maintenance at excitatory synapses than previous estimates have implied.

5.3 MOBILITY OF THE SODIUM-POTASSIUM PUMP

The proteins and supporting molecules at synaptic domains are highly dynamic and undergo rearrangement during synaptic changes. The sodium-potassium pump is responsible for maintaining the altered levels of intracellular sodium associated with changes in synaptic rearrangement, but the extent of pump mobility and dynamic regulation in neurons has not previously been addressed. With FRAP and SPT methods for measuring molecular diffusion, we detect that ATP1a3 is highly mobile in the postsynaptic membrane. Near random movement was seen in the dendrite shafts, but diffusion was more restricted at excitatory synapses contained in spines.

By altering synaptic activity levels with chemical activation or prolonged blockade of action potentials, we found that pump mobility is significantly modulated. Inhibition of signaling resulted in a reduced pump diffusion, whereas excitatory activation methods generally increased mobility. The consequence of pump mobility for sodium maintenance was measured by immobilizing the membrane bound pumps and recording sodium removal rates. We demonstrated that the rate of somatodendritic sodium removal following activity-induced sodium influx was reduced by antibody cross-linking of ATP1a3.

5.4 THE BETA SUBUNIT AS A TARGET FOR PUMP REGULATION

Functional roles of the sodium-potassium pump beta subunits in the brain are not well established. To understand the potential for mobility regulation through beta interactions, we examined the extent of alpha/beta oligomerization in the membrane. As verified by ATP1b1 co-expression studies, after insertion into the plasma membrane, pumps remain in heteromeric complexes. The large extracellular domain of the beta subunit therefore provides a potential target for modulating the pump mobility and localization.

The beta subunit-interacting protein, MONaKA, has been proposed as a regulator of the sodium-potassium pump, but little is known of its effects in living cells. We have shown that purified MONaKA, when added to culture medium, can bind to the cell surface and forms clusters along dendrites and around synapses. Application of soluble MONaKA altered the ATP1a3 trafficking in the membrane by reducing the diffusion coefficient and increasing confinement at excitatory synapse. This study suggests a novel mechanism for physiological regulation of sodium-potassium pump mobility and presents the potential for modulation of intracellular sodium maintenance via a secretory pathway.

6 FUTURE PERSPECTIVES

6.1 ALTERNATIVE THERAPEUTIC PERSPECTIVE

The description of GPCRs presented here points out a practical concern regarding the approach to treating neuropsychiatric disorders. The path exemplified by antidepressants, though effective for many suffering patients, may not be the ideal approach toward dysfunctional transmission. Attention toward postsynaptic receptor transport and efficacy could facilitate more specific regulation of signaling function and possibly reduce the unwanted side effects associated with many of the current treatments. Clearly more understanding of the mechanisms behind the problematic symptoms is needed before promising targets can be established.

6.2 ROLE OF SODIUM-POTASSIUM PUMP ISOFORMS

We have established the nanoscopic topology of the exogenous sodium-potassium pump in cultured neurons. However, fundamental questions remain that have important implications for neuronal function. One elusive task has been the topological differentiation of endogenous alpha subunit isoforms. A previous study from our group has discussed the different roles of varying isoforms, but an assessment of the actual distributions has been challenging. One limitation is the plausible abundance of the proteins in both presynaptic and postsynaptic membrane. Strategies for distinguishing between these domains while labeling endogenous protein will add useful insight to the essential function of each of the isoforms.

6.3 MOLECULAR COUNTING

Advancement of imaging methodology and analytical strategies for more accurate absolute quantification of molecules will be a major step forward for biological sciences. The optical methods discussed in this thesis provide quantification estimates that can provide valuable insight into biological processes, though practical limitations still remain. Improvement of general labeling strategies will allow investigation of endogenous protein measurements with less concern for undercounting due to incomplete labeling. Better characterization or avoidance of PAFP photoblinking will aid in improved accuracy of pointillistic counting of molecules. Protein-specific promoters should also be evaluated for more regulated expression of exogenous protein. Genetic models with knock-in small tags or PAFPs will facilitate endogenous counting for proteins lacking highly specific antibodies.

6.4 EVALUATING SYNAPTIC MOBILITY

A concern that should be more thoroughly evaluated in the future is the efficacy of labeling molecules at the synapse for SPT studies. Much of the membrane trafficking work we have presented her is based on quantum dot labeling via antibodies. As this labeling strategy requires addition of a bulky complex to the protein of interest, there are probable effects on synapse penetration. Both inhibitory and excitatory synapses

are dense with both membrane and extracellular structures that can potentially obstruct access of labeled molecules. Studies should be performed to evaluate both the effects on probability of entering synapses and the mobility within synapses. A good starting point is the combination of tracking and photoactivated localization as with SPT-PALM or similar methods such as universal PAINT (Giannone et al., 2010). These approaches, though providing data on different time scales, will allow us to evaluate the impact of method selection on measured diffusion characteristics.

6.5 MONAKA IN THE BRAIN

This study presents MONaKA as a promising modulator of the sodium-potassium pump. Details of protein expression and transport/secretion are still enigmatic, but the exciting potential of MONaKA warrants future investigation to develop further understanding. Revelation of secretory mechanisms and sites of release from neurons containing endogenous MONaKA has yet to occur. Understanding the regulation mechanisms will help describe the physiological role of this protein and may lead to novel and impactful therapeutic modulators of sodium-potassium pump function.

6.6 CULTURED NEURONS VS. INTACT BRAIN

A final thought on future perspectives relates both to the relevance and applicability of the used methods in the native environment of neurons. As we have focused exclusively on cultured neurons, we must be aware of inherent differences in the study model. Though we have optimized maturity and development of our cultured neurons, there are expected differences from the complex environment of the brain. The intact brain contains structured networks of neurons that are too complex to generate in primary culture. Another inherent difference is the three dimensional structure of brain tissue that includes extensive matrix molecules and a variety of regulated cell types. Primary culture is a different environment that can impact cell function in unknown ways. The results of the trafficking studies presented here should be examined in more complex systems to validate the relevance.

This, however, leads to the concern of method applicability. The common trafficking methods discussed here have not been widely applied to intact brain tissue due to numerous technical limitations. One principal challenge is effective targeting of molecules deep in tissue or even in more superficial layers. Antibody-based methods such as QD SPT are not feasible because of futile labeling. Additionally, super-resolution imaging methods are greatly hindered in tissue. Though tremendous improvements have been presented in the last few years that allow imaging of the intact brain, there is still a limitation for live cell imaging. Accordingly, single molecule trafficking methods in tissue are practically unattainable. Further methodological advancements that will enable effective SPT-PALM or novel SPT approaches will be remarkable breakthroughs that will allow us to validate the details we have acquired in the last decade from primary culture studies.

7 ACKNOWLEDGEMENTS

This work is a product of contributions from many people.

To my supervisors:

My main supervisor, **Dr. Hjalmar Brismar**; For exposing me to this fascinating world of optical microscopy. Thank you for investing in me during both my Master's and PhD education. I am grateful for your support and the provision of these cutting edge tools that any imaging biologist dreams of. For sharing insight into effective experimental strategy and for promoting me along the way.

Dr. Anita Aperia; For your endless efforts to make sense of the cumbersome results we have waded through over the years. Your tireless dedication to enhancement of this work was matched only by your overall passion for science. Thank you for shaping our ideas into messages worthy of sharing with the community and for repeatedly teaching the essential art of scientific communication.

Dr. Per Svenningsson; For your guidance and constant encouragement. You allowed me to walk in the footsteps of your inspiring work and always prioritized me. Thank you for always providing advice and support without hesitation.

To my co-authors:

Dr. Markus Kruusmägi; It is difficult to measure the contributions from my close colleague. I can say without ambiguity that you showed me the ropes of advanced microscopy. You were there every step of the way and helped me to produce the best possible imaging throughout my PhD. As if this wasn't enough, you helped me to climb and conquer the ropes of programming and image analysis. This thesis work is a product of your dedication and support.

Dr. Lena Scott; Lena, you are the essence of cool and collected in the midst of the chaos of research. From my first days as a PhD student, you patiently and painstakingly introduced me to a number of methods in the lab. You have always been available and willing to assist me with my numerous difficulties, and I will always be appreciative.

Dr. Alexander Bondar; For your admirable example of dedication, thoroughness and openness. You taught me the value of a strong work ethic and gave me the means to be extremely creative and productive with DNA construction.

Dr Hans Blom; For your support and encouragement with super-resolution microscopy. I am grateful for your invaluable assistance and teachings, which were even more frequent than the times you referred to my PALM imaging as 'blinky-blink.' I hope to have absorbed even a fraction of your impressive attention to details and competence in microscopy.

Dr. Nermin Sourial-Bassilious; Thank you for lending your skills in biochemistry and your endless determination. You are the model of patience and perseverance.

Dr. Paul Greengard; It was a pleasure and honor to work with you and experience your approach to unwrapping the mysteries of science. I will always appreciate the scientific and social interaction and hope for future opportunities to expand upon both.

Dr. Marc Flajolet; For setting aside your precious time to work at the lab bench with me in New York. Your consistent support was a source of encouragement and motivation throughout our collaboration.

To others for your contributions to this work:

Dr. Antoine Triller and **Dr. Marianne Renner**; For guiding me over the first hurdles of single particle tracking. The discussions and feedback were immensely constructive. Thank you for experimental support and for the elaborate and sophisticated analytical software that allowed us to dig deeper into the world of lateral diffusion.

Linda Westin; For your perpetual support with maintaining quality cultures and the willingness and eagerness to share conceptually in this common search for truth. Your unwavering commitment to science never ceases to impress me.

To a select few who continually provided invaluable support without due credit:

Lill-Britt Svensson; For your support with essentially everything in the lab. You have always been a strength of the lab, and your willingness to assist with any and every task is testament to your generosity.

Raija Wallenborg; For your constant awareness of all necessities I have managed to neglect throughout my PhD. Your steadfast attention has undoubtedly eliminated many potential difficulties.

Josephine Forsberg; For your skill and professionalism that was available at a moment's request. Your persistent support to everyone in our group was more impactful than you can appreciate.

None of you three have expected or received just acknowledgement for your steadfast efforts. Know that you are inspirational people and models of selflessness. If I had the talent to write a tribute song for you, it would be exactly the opposite of Sammy Hagar's "I've done everything for you."

To all my current and former colleagues at KI, KTH, and SciLifeLab:

Zuzana Spicarova Khorshidi and **Nina Illarionova** for arranging lively social events and assisting in the lab. **Dr. Yutong Song** for keeping me on my toes (and batteries out of my computer mouse). **Dr. Jacopo Fontana** for all the fun game nights and great dinners. **Dr. Matthias Reuss** for simultaneously educating me in world-class soccer and world-class imaging. My thanks to **Dr. Guillaume Azarias**, **Kristoffer Bernhem**,

Evgeniya Burlaka, **Dr. Siobhán Connor**, **Dr. Susanne Crambert**, **Dr. Nicolas Fritz**, **Dr. Ulla Holtbäck**, **Dr. Georgiy Khodus**, **David Larsson**, **Dr. Xiao Li Lu**, **Dr. Otto Manneberg**, **Eivor Zettergren Markus**, **Linnea Nilsson**, **Panos Papachristou, Dr. Carolina Rigos, Dr. Stefan Wennmalm** and **Dr. Hatti Qin** for making it a little easier to be away from home for so long. **Dr. Evgeny Akkuratov** for your scientific discussion. **Dr. Jacob Kowalewski** for being a supportive colleague and sharing your expertise in trafficking experimental and analytical methods. To **Athanasia Christakou** for always looking out for me and being an exceptional friend. A special thank you to **Dr. Şermin Tükel** for being a creative inspiration. **Dr. Ali Khorshidi** for your eternal enthusiasm. **Erland Lewin** and **Dr. Susanna Rydholm** for being perfect colleagues and helping to get me started with my research career. To **Georgios Alexandrou** for being the best Christmas host. **Per Olofsson**, for often checking 'what's up' in the late working hours. **Sahar Ardabili**, for being a kind friend and the perfect lunch buddy.

Dr Shun Yu. I am grateful for an endless list of thing. I will always remember your friendship and encouragement. I cherish the experiences we have shared, both here in Sweden and during the trips to China and Germany.

To my dear friend **Dr. Padideh Kamalizare**. You are greatly missed in Sweden. I fondly remember our times at Cell Physics and am thrilled to finally have more chances to visit.

The one and only **Dr. Zachi Horn**. Sweden just isn't the same without you. Thanks for all the great times, and I'm looking forward to many more back in the states.

The amazing **Dr. Eli Gunnarson** for always caring and for sharing your precious time. **Dr. Gerald DiBona**, thank you for being a source of wisdom and a generous friend. To **Dr. Sergey Zelenin** and **Dr. Marina Zelenina** for all the support, in and out of the lab.

Dr. Yuri Shvarev, thank you for always making a point to stop by and catch up each time you are away (and always bringing Russian chocolates!). **Dr. Björn Önfelt**, the epitome of laid-back. **Dr. Aman Russom**, thank you for always waiting for me to show up for posters with a smile and, more importantly, a coffee.

To all my beloved friends in Sweden:

You are too numerous to mention, but know that you are my extended family. I am saddened to leave you, but I will fondly remember the incredible experiences we have shared. I will always be waiting with open arms just a flight away.

And finally, I want to dedicate this work to my family. Thank you for all the love and support, even from this long distance.

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